VOLUME XXXIV AUGUST, 1955 NUMBER 8

The Journal of Clinical Investigation

EDITED FOR THE AMERICAN SOCIETY FOR CLINICAL INVESTIGATION

Contents

Effect of Penicillin on Group A Streptococci *In Vivo* in the Absence of Leucocytes. JAMES E. DARNELL, JR., BARBARA B. PESCH, AND ROBERT J. GLASER 1237

Estimation of the Size of the Patent Ductus Arteriosus from Hemodynamic Data. NOBLE O. FOWLER AND EDGAR P. MANNIX 1242

Renal Clearance of Eighteen Individual Amino Acids in Human Subjects. PAUL D. DOOLAN, HAROLD A. HARPER, MAXINE E. HUTCHIN, AND WALTON W. SHREEVE 1247

Studies on Ketone Metabolism in Man. I. A Method for the Quantitative Estimation of Splanchnic Ketone Production. EMILE E. WERK, JR., HARRY T. MCPHERSON, LADD W. HAMRICK, JR., JACK D. MYERS, AND FRANK L. ENGEL 1256

Abnormalities of Carbohydrate Metabolism of Red Cells in Hereditary Spherocytosis. THOMAS A. J. PRANKERD, KURT I. ALTMAN, AND LAWRENCE E. YOUNG 1268

The Effect of Acute Respiratory Acidosis on the Internal Equilibrium of Potassium. BELDING H. SCRIBNER, KENNETH FREMONT-SMITH, AND JAMES M. BURNELL 1276

Estimation of the Phospholipid Phosphorus Turnover Time in Man: Studies in Normal Individuals, in Patients with the Nephrotic Syndrome and in Other Types of Hyperlipemia. HUGO W. MOSER AND KENDALL EMERSON, JR. 1286

The Effects of Mitral Valvuloplasty on Cardiovascular and Renal Function at Rest and During Exercise. WALTER E. JUDSON, J. D. HATCHER, WILLIAM HOLLANDER, AND MEYER H. HALPERIN 1297

Conditions of Formation of Euglobulin-Like Precipitates from Serum Proteins and Chondroitin Sulfate. JACQUES BADIN AND MAXWELL SCHUBERT 1312

Plasma Polysaccharide Fraction Containing Uronic Acid, in Normal Subjects and in Patients with Rheumatoid Arthritis. JACQUES BADIN, MAXWELL SCHUBERT, AND MARY VOURAS 1317

Thyroxine-Binding Capacity of Serum in Normal Man. JACOB ROBBINS AND J. E. RALL 1324

Effects of Triiodothyronine and Other Thyroxine Analogues on Thyroxine-Binding in Human Serum. JACOB ROBBINS AND J. E. RALL 1331

Partial Characterization of Urinary Adrenocortical Steroids in Adrenal Hyperplasia. WALTER R. EBERLEIN AND ALFRED M. BONGIOVANNI 1337

The Journal of Clinical Investigation

Printed by the Lancaster Press, Inc., Prince and Lemon Sts., Lancaster, Pa. Published by the American Society for Clinical Investigation. Publication Office, 622 West 168th St., New York 32, N. Y.

STANLEY E. BRADLEY, EDITOR-IN-CHIEF

ASSOCIATE EDITORS

LUDWIG W. EICHNA IRVING M. LONDON

The Editorial Committee of the American Society for Clinical Investigation

THOMAS P. ALMY, *New York, N. Y.*
S. HOWARD ARMSTRONG, JR., *Chicago, Ill.*
ROBERT W. BERLINER, *Bethesda, Md.*
FRANCIS P. CHINARD, *Baltimore, Md.*
WILLIAM J. DARBY, *Nashville, Tenn.*
DAVID P. EARLE, JR., *Chicago, Ill.*
RICHARD V. EBERT, *Little Rock, Ark.*
LEONARD P. ELIEL, *Oklahoma City, Okla.*
WARD S. FOWLER, *Rochester, Minn.*
F. RAYMOND KEATING, JR., *Rochester, Minn.*
CHARLES H. RAMMELKAMP, *Cleveland, O.*
EDWARD C. REIFENSTEIN, JR., *Butler, N. J.*
I. HERBERT SCHEINBERG, *New York, N. Y.*
RICHARD W. VILTER, *Cincinnati, O.*
LAWRENCE E. YOUNG, *Rochester, N. Y.*

THE JOURNAL OF CLINICAL INVESTIGATION is designed for the publication of original investigations dealing with or bearing on the problems of disease in man, and it is the policy of the editors that the JOURNAL should cover the field of clinical investigation in its broadest sense.

Contributions should be sent to the **Editor-in-Chief, Dr. Stanley E. Bradley, 622 West 168th St., New York 32, N. Y.** In order to hasten the process of reviewing and editing it is requested that each original manuscript be accompanied by a duplicate copy and duplicate illustrations.

In order further to expedite the earliest possible publication of articles submitted to the JOURNAL OF CLINICAL INVESTIGATION they will be published in the order of final processing after review, correcting and editing rather than in the strict sequence of their receipt in this office. Papers which require much editorial change, revision of charts and tables, condensation and rewriting will be delayed according to the time required for their correction. This policy will put a premium on excellence of manuscript as well as the major consideration of content. Because of the increasing costs of printing, especially in the hand work of making tables, the editors suggest that when feasible photographs of tables be prepared. It is also requested that charts be photographed, the photographs accompanying the manuscript. The originals of the charts need not be forwarded except by special request of the editors. Further information regarding the preparation of manuscripts may be obtained from the editors.

Each volume will consist of 12 monthly issues appearing throughout the calendar year. The subscription price is $12 per volume, **payable in advance;** foreign countries also $12, payable in U. S. funds. Single copies will be sold when available at Vols. 1–28, $2; Vols. 29–31, $1.50 a copy. Authors may purchase reprints at scheduled prices. **A special subscription rate of $6 per volume of 12 monthly issues is offered to medical students, interns, fellows, and residents of approved hospitals.** Subscriptions are **net postpaid.** Remittance should be made by draft, check or postal money order, payable to **The Journal of Clinical Investigation, 622 West 168th St., New York 32, N. Y.** Claims for copies lost in the mails must be received within **thirty** days (domestic), **ninety** days (foreign), of the date of issue. Changes of address must be received at least two weeks in advance of issue.

Acceptance for mailing at special rate of postage provided for in paragraph (d-2), section 34.40, P.L. & R. of 1948, authorized February 13, 1950

Entered as second-class matter September 16, 1931, at the Post Office at Lancaster, Pa., under the Act of March 3, 1879

Copyright, 1955, American Society for Clinical Investigation

New, Well Tolerated Medium for Excretory Urography

94%

DIAGNOSTIC FILMS

in a series of 1123 patients

Hypaque® SODIUM

50% solution

Write for detailed literature or consult your local Winthrop-Stearns' representative.

Winthrop-Stearns INC.

NEW YORK 18, N. Y. WINDSOR, ONT.

Hypaque sodium, brand of diatrizoate sodium (sodium 3,5-diacetamido-2,4,6-triiodobenzoate)

for the patient with fever,

SF*

means (*Pfizer*) antibiotics, *fortified with vitamins*

to support recovery, speed convalescence

Tetracyn® SF*

BRAND OF TETRACYCLINE

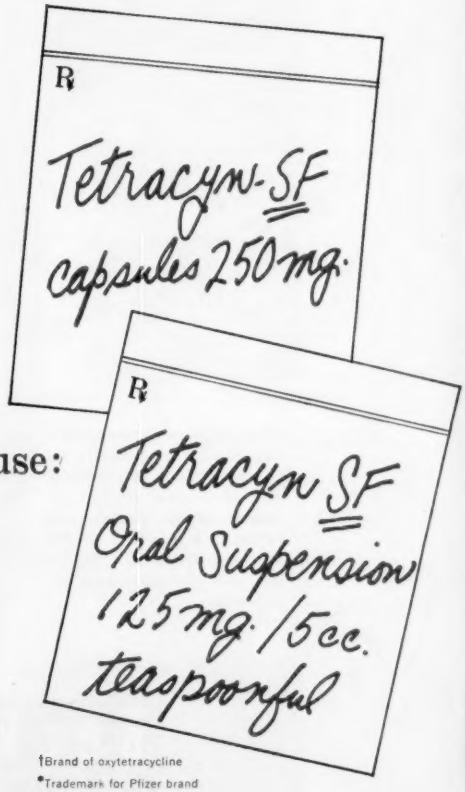
the leading broad-spectrum antibiotic, discovered by Pfizer

with water-soluble vitamins in combinations originated by Pfizer

For patients with infections, "one must aim at maintaining the normal daily nutritional requirements, replacing previous depletions and current losses, and supplying whatever increased requirements may be related to the nature of the illness."[1] This is the concept of "treating the 'whole' patient."[2]

Tetracyn SF has antibiotic effectiveness equal to that of Tetracyn® alone[3] and, in the hands of thousands of physicians, has shown

Equivalent Blood Levels[3]
Superior Toleration[4]
Accelerated Recovery[5]



Two effective dosage forms for oral use:

Terramycin† SF* ***is also available.***

Tetracyn SF and Terramycin SF are formulated to provide with the minimum daily dose of each antibiotic (1 Gm. of Tetracyn or Terramycin) the stress vitamin formula recommended by authorities on nutrition.[1]

1. Pollack, H., and Halpern, S. L.: Therapeutic Nutrition, Prepared in Collaboration with the Committee on Therapeutic Nutrition, Food and Nutrition Board, National Research Council, Washington, D. C., 1952. 2. Martí-Ibáñez, F.: Antibiotic Med. *1*:247 (May) 1955. 3. Dumas, K. J.; Carlozzi, M., and Wright, W. A.: Antibiotic Med. *1*:296 (May) 1955. 4. Milberg, M. B., and Michael, M., Jr.: Ann. New York Acad. Sc., In press. 5. Prigot, A.: Ibid.

†Brand of oxytetracycline

*Trademark for Pfizer brand of antibiotics with vitamins.

Pfizer **PFIZER LABORATORIES,** Brooklyn 6, N.Y.
Division, Chas. Pfizer & Co., Inc.

faster and easier **Na** and **K** determinations

with the BECKMAN direct-reading flame photometer

This modern Flame Photometer makes sodium and potassium determinations faster than ever before! Reads directly in milliequivalents without curves or tables. The answer you get is the answer you want.

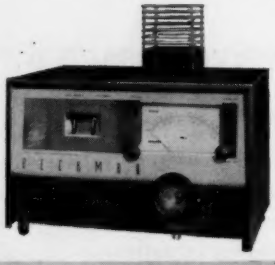
A unique double control eliminates preliminary fussing. One lever selects the proper potentiometers and filters for either sodium or potassium readings. A pilot light and preset adjustments give instantaneous and efficient flame operation.

To provide long life and trouble-free performance, this Photometer has a stainless steel atomizer separate from the burner. A nebulizing chamber shoots only finely atomized sample to the flame. This reduces internal moisture condensation and clogging deposits, and maintains the flame background noise at a low and steady level.

Natural gas or propane, a source of compressed air, and household current make the Direct-Reading Flame Photometer instantly ready to operate.

The Beckman Direct-Reading Flame Photometer is ideally suited for sodium and potassium studies in human serum, blood, and urine. It is sensitive to .02 milliequivalents of sodium and .002 milliequivalents of potassium.

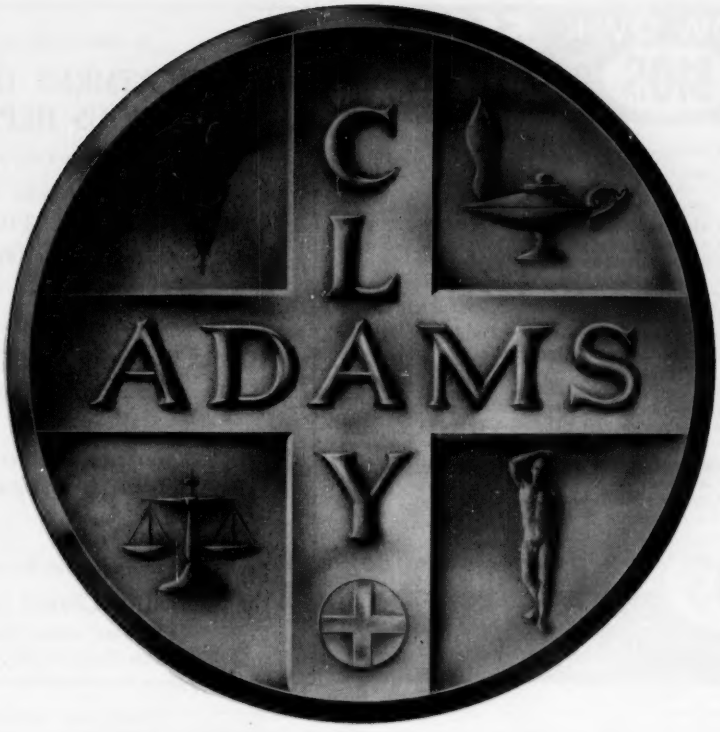
See your Beckman dealer or write for Data File 37-76



Beckman *division*

BECKMAN INSTRUMENTS, INC.

FULLERTON 1, CALIFORNIA



Depend on Clay-Adams...

The *preferred* source of supply for leading hospitals, laboratories and teaching institutions Quality products through leading surgical and scientific dealers everywhere. Our 216-page catalog describes 1161 products.

Look for these familiar trade names – your assurance of quality!
Yankee • Gold Seal • Adams • Medichromes • Autoclips • CRI Intramedic • Durable • Cantor • Kahn

Clay-Adams **NEW YORK 10**

NOW OVER 5100
BIOS CHEMICALS

- Calcium Glycocholate
- Calcium Malate
- Calcium Metaborate
- Calcium α-Naphthylphosphate
- Calcium Sucrate
- Calcium Undecylenate
- Camphoquinone
- Camphor Salicylate
- Camphylamine
- Cantharidin
- p-Carbamylphenylarsine Oxide
- Carbobenzoxy-1-glutamyl-1-tyrosine
- Carboxybenzenesulfonamide
- p-Carboxyphenylarsinoxide
- 5-Carboxyuracil (Uracil-5-carboxylic acid)
- Carnaubic Acid
- Carnaubyl Alcohol
- Caronamide
- Carragheenic Acid

Ask for our new complete catalogue

BIOS Laboratories, Inc.
17 West 60th St. New York 23 N. Y.
PLaza 7-8171

Single Copies of

THE TREATMENT OF ACUTE INFECTIOUS HEPATITIS

July, 1955, Part Two issue of

THE JOURNAL OF CLINICAL INVESTIGATION

are available for $1.50

Preprints of the

PROGRAM of the FORTY-SEVENTH Annual Meeting

of the

American Society for Clinical Investigation

$1.00

Order both through the Business Office of

The Journal of Clinical Investigation
622 West 168th Street
New York 32, N. Y.

CLINICAL SCIENCE

Edited for the Medical Research Society by
W. MELVILLE ARNOTT

Assistant Editors
G. D. DAWSON, K. W. DONALD, H. E. DE WARDENER

Advisory Board

E. A. CARMICHAEL
R. V. CHRISTIE
R. T. GRANT
J. McMICHAEL
G. W. PICKERING
E. E. POCHIN
E. P. SHARPEY-SCHAFER
JANET VAUGHAN
W. C. WILSON
L. J. WITTS

Volume 14, No. 3. **Issued August 1955**

Asymmetry of Blood-flow in the Feet in Anterior Poliomyelitis. By A. MCPHERSON and A. W. L. KESSEL.
The Uptake of Radioactive Phosphate and Its Distribution Amongst Esters in Sickled Cells. By T. A. J. PRANKERD.
Observations on the Relationship of Intrinsic Factor to the Absorption of Labelled Vitamin B_{12} from the Intestine. By SHEILA T. CALLENDER and J. R. EVANS.
The Absorption and Utilization of Radioiron in Rheumatoid Disease. By M. R. JEFFREY, H. F. FREUNDLICH, E. B. JACKSON and D. WATSON.
Changes in Splanchnic Blood Volume and Splanchnic Blood Flow in Dogs after Haemorrhage. By P. C. REYNELL, P. A. MARKS, C. CHIDSEY and S. E. BRADLEY.
Acidosis in Experimental Electrolyte Depletion. By EDWIN CLARKE, BARBARA M. EVANS, I. MACINTYRE and M. D. MILNE.
Variations in the Iodine Avidity of the Normal Human Thyroid as Measured by the 24-hour ^{131}I Uptake. By E. H. HARE and C. P. HAIGH.
Cellular Cation Changes in Rat Muscle Following Ischaemic Anoxia and Trauma. By IAN W. MACPHEE.
A Study of Arterial Blood Pressure and Its Inheritance in a Sample of the General Population. By W. E. MIALL and P. D. OLDHAM.
The Effect of A.T. 10 on Calcium and Phosphorus Metabolism in Resistant Rickets. By PAUL D. SAVILLE, J. R. NASSIM, F. HARWOOD STEVENSON, LILY MULLIGAN, and MARGARET CAREY.
Further Observations on the Distribution of Red Cells and Plasma in Disease—the Low Body Haematocrit: Venous Haematocrit Ratio. By A. HOPE and D. VEREL.
The Relationship Between Plasma Albumin Concentration and Protein Excretion in Patients with Proteinuria. By J. HARDWICKE and J. R. SQUIRE.
Changes in the Oxygen Content of Axillary Venous Blood During Leg Exercise in Patients with Rheumatic Heart Disease. By K. W. DONALD, J. M. BISHOP and O. L. WADE.
The Quantitative Estimation of Valvular Incompetence by Dye Dilution Curves. By P. I. KORNER and J. P. SHILLINGFORD.

Subscription Rate: £3.15.0. per volume.

Publishers: Shaw & Sons Ltd., 7, 8 & 9, Fetter Lane, Fleet Street, London, E. C. 4, England

Upjohn

Bacterial diarrheas...

Kaopectate

Trademark, Reg. U.S. Pat. Off.

with Neomycin

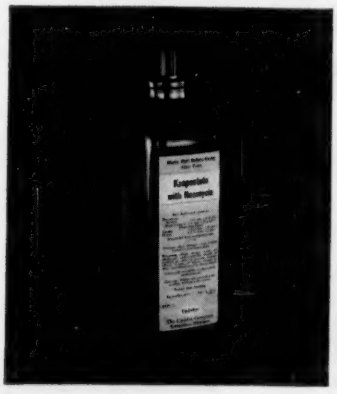
Each fluidounce contains:

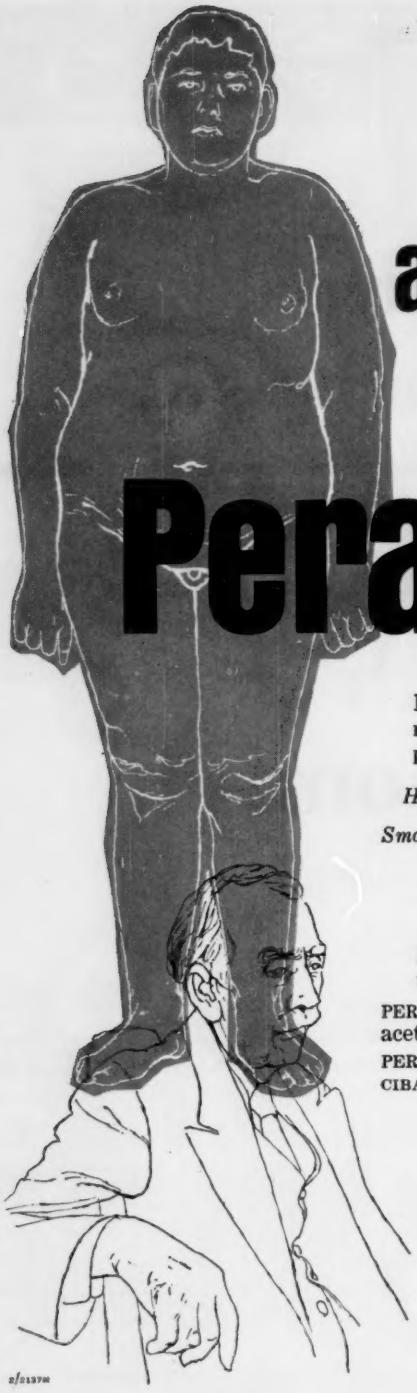
Neomycin sulfate . 300 mg. (4⅔ grs.)
[equivalent to 210 mg. (3¼ grs.) neomycin base]
Kaolin . . . . . 5.832 Gm. (90 grs.)
Pectin . . . . . 0.130 Gm. (2 grs.)
Suspended with methylcellulose 1.25%

Supplied:

6-fluidounce and pint bottles

The Upjohn Company, Kalamazoo, Michigan





stronger sustained androgenic activity

Perandren®

phenylacetate

In addition to Perandren® propionate, CIBA now offers the longer-acting Perandren phenylacetate. Its advantages:

•

High Levels of Androgenic and Anabolic Effect

•

Smooth Continuous Action—No Peaks and Valleys

•

Virtually Painless Injection

•

One Dose Usually Effective for a Month

Multiple-dose vials, 10 ml., each ml. containing 50 mg. Perandren phenylacetate.

PERANDREN® phenylacetate (testosterone phenylacetate CIBA)

PERANDREN® propionate (testosterone propionate CIBA)

CIBA
SUMMIT, N. J.

EFFECT OF PENICILLIN ON GROUP A STREPTOCOCCI *IN VIVO* IN THE ABSENCE OF LEUCOCYTES[1]

By JAMES E. DARNELL, JR.,[2] BARBARA B. PESCH, AND ROBERT J. GLASER

(From the Division of Immunology, Department of Internal Medicine, Washington University School of Medicine, St. Louis, Mo.)

(Submitted for publication March 8, 1955; accepted March 23, 1955)

Studies of penicillin action *in vitro* have indicated that, under given experimental conditions, the antibiotic is bactericidal rather than bacteriostatic (1–5), and it has been further demonstrated that it exerts its bactericidal effect on susceptible microorganisms only when they are actively metabolizing, a state indicated usually by rapid growth (6–9). Although there is also evidence that when it is administered in the initial phase of experimental infections due to susceptible organisms penicillin is bactericidal (10, 11), elucidation of its effect *in vivo* has been complicated by the participation of host cellular defenses which, under certain circumstances, may in themselves be extremely efficient (12). The recent description by Werner, Knight, and McDermott of a method by which bacteria can be localized *in vivo* in a cell-free milieu (13) suggested the means by which the effect of penicillin on group A streptococci, in the absence of leucocytes, could be assayed at various stages of growth in the peritoneal cavity of the rabbit.

METHODS AND MATERIALS

Preparation of agar disks. The techniques employed were patterned after those described by Werner, Knight, and McDermott (13). A 2 per cent suspension of nonnutrient agar (Difco Bactoagar) in distilled water at pH 7.8 was sterilized in flasks in the autoclave and stored at 2° C. The disks were made by pouring melted agar, at 39° C to 41° C, under aseptic precautions, into thin-walled cylindrical aluminum molds measuring 22 mm. in diameter and 15 to 20 mm. in height. The sterile cylinders were placed in petri dishes and a 1 ml. layer of agar introduced. The petri dish was then covered and cooled at 2° C for 15 minutes. The second layer, containing the inoculum in 0.2 ml. of agar, was poured onto the hardened first layer, care being taken to prevent any of the bacteria-containing suspension from reaching the wall of the mold; the desired result was easily achieved because the 0.2 ml. layer solidified almost immediately on contact with the hardened basal portion. Two ml. of plain agar were added as a cover to complete the disk, and after the agar had hardened, the aluminum ring was removed.

Preparation of inoculum. In the experiments herein described, a single strain of group A streptococcus, S23 Burbank, was employed. The organism was obtained originally from Dr. Maclyn McCarty of the Rockefeller Institute for Medical Research; identical stock samples were prepared by concentrating cultures of the organism, grown in beef infusion broth with 10 per cent sheep serum and 0.5 per cent dextrose (BIB-SD), and storing 0.2 ml. aliquots at −72° C. For the various studies described, 5 ml. of BIB-SD at pH 7.8 were inoculated with 2 loopfuls of rapidly thawed stock culture and incubated at 37° C for 16 hours, after which 0.5 ml. was transferred to 5 ml. of fresh BIB-SD and incubated for 4 hours. Ten-fold dilutions were made in tryptose phosphate broth, and 1.0 ml. of the 10^{-6} dilution was added to 9.0 ml. of melted agar at 40° C and thoroughly mixed. The 0.2 ml. portion of this mixture, employed in the disk, contained 350 to 400 chains.

Implantation of disks. New Zealand rabbits, obtained from a single breeder, and free of demonstrable infection were used. The animals weighed 2.5 to 3.0 Kg., and were maintained under standard laboratory conditions. Preoperative preparation included careful depilatation of the abdomen over a rectangular area approximately 12 × 8 cm.; this area was then scrubbed vigorously with hexachloroprene soap (pHisoderm®). Laparotomy was performed under local anesthesia with 1 per cent procaine hydrochloride; aseptic technique was employed. A 6 to 8 cm. midline incision was made, and the disks placed so that contact between their surfaces was prevented. It was possible to insert as many as six disks on either side of the peritoneal cavity without difficulty. After implantation was completed, the peritoneum and muscle layers were closed with a continuous silk suture and the skin with Michele clips.

Disks were removed through the same incision, and ground separately, without sand, in sterile mortars. In order to bring the final volume to 8.0 ml., 4.8 ml. of tryptose phosphate broth were added, and poured blood agar plates were made with 1 ml. aliquots of suitable ten-fold dilutions of the ground disk suspensions for determination of the number of viable chains of streptococci.

Administration of penicillin. In all of the experiments, a standard dose of 30,000 units of aqueous procaine penicillin G was employed and the drug was administered

[1] Supported by a grant from the Life Insurance Medical Research Fund.

[2] Fourth year medical student when this work was done.

intramuscularly according to the schedules described in the following section.

When penicillin was administered prior to disk removal, 10 to 20 units of penicillinase (Schenley) were incorporated in the poured plates made from such disks; these amounts were in excess of those needed to inactivate the drug in the concentrations present.

Penicillin content of agar disks was determined at various intervals, including those instances in which prior observations indicated that viable bacteria persisted despite exposure to the antibiotic. Qualitative assays were performed by the serial dilution capillary tube method of Fleming (14). Disks were ground in a sterile mortar, and 4.8 ml. of tryptose phosphate broth were added, making a final volume of 8.0 ml. Aliquots of this suspension were used for the penicillin determinations.

RESULTS

General observations. Contamination of the peritoneal cavity by streptococci did not occur except in a single instance when a disk was inadvertently fractured during removal. Although a fibrin-leucocyte membrane formed about disks in approximately 24 hours, disk integrity was maintained, and leucocytes did not gain access to the agar. The latter findings confirm those of Werner, Knight, and McDermott (13).

Normal growth curve. After intraperitoneal implantation in disks, the streptococci entered the logarithmic phase of growth immediately, and continued to multiply at a maximal rate for eight hours (Figure 1), attesting to the prompt diffusion of essential nutrients from the host environment into the disk. Thereafter only a slight increase in bacterial population was noted, and a plateau was

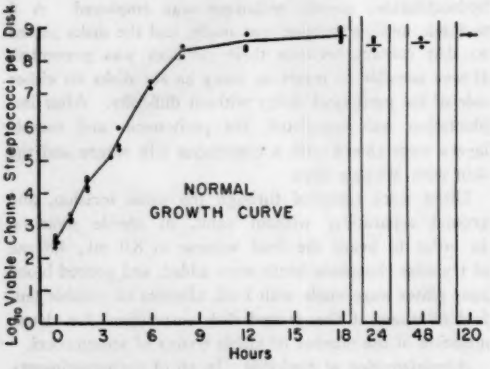


FIG. 1. NORMAL GROWTH CURVE FOR STREPTOCOCCAL STRAIN S23 BURBANK WHEN IMPLANTED INTRAPERITONEALLY IN DISKS

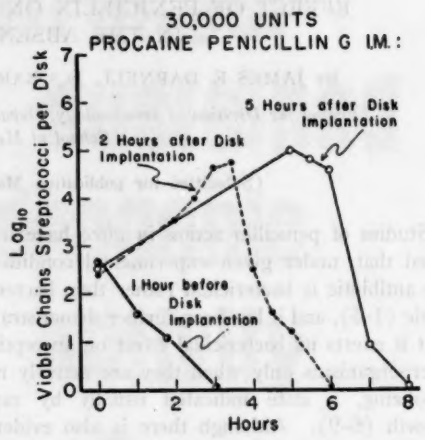

30,000 UNITS
PROCAINE PENICILLIN G I.M.:
5 Hours after Disk Implantation
2 Hours after Disk Implantation
1 Hour before Disk Implantation
Log10 Viable Chains Streptococci per Disk
Hours

FIG. 2. BACTERICIDAL EFFECT OF PENICILLIN, GIVEN BEFORE OR SOON AFTER DISK IMPLANTATION, ON STREPTOCOCCI

reached by eighteen hours. Little change in the bacterial count was noted for the next five days after which the viable organisms gradually diminished in number, and five weeks after implantation the disks were sterile. The growth curve for the strain employed was readily reproducible as is indicated by the fact that the multiple coordinates for any given time shown in Figure 1 represent observations made on disks from different animals.

Effect of single doses of penicillin. In various experiments single injections of penicillin were given rabbits prior to or at intervals after implantation of disks. It may be seen in Figures 2, 3, and 4 that three kinds of effects were noted after administration of single doses of the antibiotic. Thus when the bacteria were exposed to penicillin within five hours of disk implantation (Figure 2) sterilization was achieved in the ensuing four hours. On the other hand, when treatment was not instituted until 12 hours after the disks were introduced into the abdominal cavity (Figure 3) the bacterial population was affected as indicated by a decrease in the number of viable chains, but sterilization could not be attained. Finally, penicillin given 24 hours after disk implantation, after the streptococci had reached the plateau phase in their growth, failed to effect any reduction in the number of viable bacteria (Figure 4).

Effect of multiple doses of penicillin. In view of the fact that single large doses of penicillin

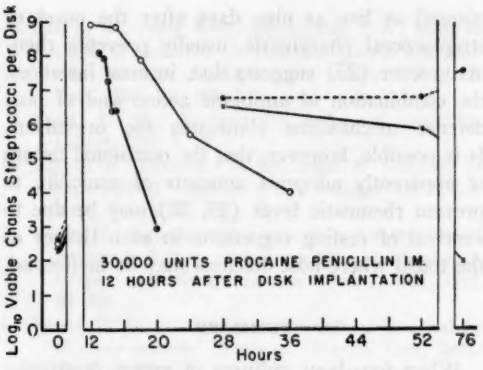


FIG. 3. PARTIAL KILLING OF STREPTOCOCCI BY PENICILLIN ADMINISTERED 12 HOURS AFTER DISK IMPLANTATION

Each curve represents a separate experiment.

failed to sterilize disks in which growth of streptococci had proceeded for 12 or more hours, it was thought desirable to ascertain the effect of multiple doses of penicillin under comparable conditions. In separate experiments, therefore, disks were placed intraperitoneally in three rabbits and penicillin was given 24, 48, and 72 hours later. Even this intensive therapeutic regimen failed to kill all of the streptococci (Figure 5), although there was apparently a significant decrease in the number of viable bacteria.

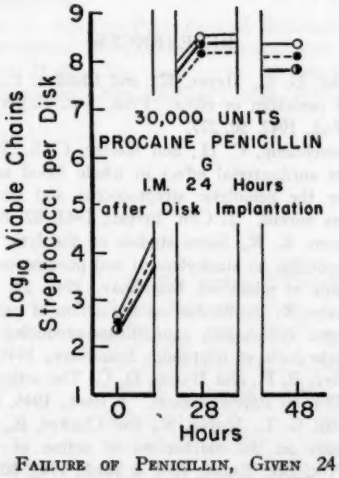


FIG. 4. FAILURE OF PENICILLIN, GIVEN 24 HOURS AFTER DISK IMPLANTATION, TO INFLUENCE VIABILITY OF STREPTOCOCCI

Each curve represents a separate experiment.

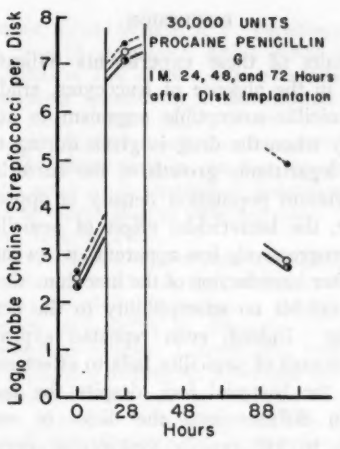


FIG. 5. FAILURE OF PENICILLIN, WHEN GIVEN IN REPEATED DOSES BEGINNING 24 HOURS AFTER DISK IMPLANTATION, TO EFFECT STERILIZATION OF THE FOCI

Each curve represents a separate experiment.

Penetration of penicillin into disks. The results of determination of penicillin content of disks at varying intervals after drug administration are given in Table I. They indicate that the failure of penicillin to kill organisms 12 or more hours after implantation was not due to impenetrability of the disk to the drug; indeed, the amount of drug which diffused into the disk was of an order which would have been bactericidal for rapidly growing cultures of the organism employed in the experiments.

TABLE I

Penetration of Agar Disks by Penicillin

Interval in Hours Between Disk Implantation and Penicillin Administration	Interval in Hours Between Penicillin Administration and Disk Assay	Result of Penicillin Assay
2	2	+
	4	+
	6	+
	12	+
	24	0
12	3	+
	6	+
	12	+
24	4	+
	28	0

+ indicates presence of, and 0 absence of, penicillin in bacteriostatic amounts. It should be noted that the penicillin present in the disk (volume 3.2 ml.) was diluted to a final volume of 8.0 ml. before the determinations were made.

DISCUSSION

The results of these experiments indicate that *in vivo,* in the absence of leucocytes, eradication of a penicillin-susceptible organism is achieved promptly when the drug is given during the period of logarithmic growth of the bacteria. As the maximum population density is approached, however, the bactericidal effect of penicillin becomes progressively less apparent and twenty-four hours after introduction of the inoculum, the streptococci exhibit no susceptibility to the action of the drug. Indeed, even repeated exposure to large amounts of penicillin fails to effect sterilization of the bacterial foci, despite the fact that penicillin diffuses into the disks in sufficient amounts to kill rapidly multiplying organisms. The findings thus confirm earlier *in vitro* (1–5) as well as *in vivo* observations (10, 11), and suggest that the favorable outcome usually obtained in the treatment of patients with group A streptococcal infections is due in considerable part to the host cellular defenses, participation of which was excluded in the studies herein described. It may also be inferred that the previously demonstrated inadequacy of short-term penicillin therapy (15, 16) results from persistence of resting streptococci in foci, such as abscesses, in which host defenses are hampered (17). It is well known that group A streptococci may be carried in the pharynx for long periods of time (18, 19), often in a metabolically altered state (20). That the organisms may be recovered from the excised tonsils of patients subjected to tonsillectomy, even when pharyngeal cultures have failed to reveal the presence of streptococci, has been repeatedly demonstrated (21, 22). It is of considerable importance that in some of these instances penicillin therapy had been given in the immediate pre-operative period (23, 24). Since most of the patients from whose tonsils streptococci were recovered had no clinical findings suggestive of pharyngitis it can be presumed that they were carriers of the organisms.

Since it has been postulated that in man persistence of streptococci may be of importance in the pathogenesis of rheumatic fever (25, 26), this study re-emphasizes the importance of early penicillin treatment of streptococcal infections (27). The fact that intensive penicillin therapy, instituted as late as nine days after the onset of streptococcal pharyngitis, usually prevents rheumatic fever (25) suggests that, in most instances, the combination of antibiotic action and of host defense mechanisms eliminates the organisms. It is possible, however, that the occasional failure of apparently adequate amounts of penicillin to prevent rheumatic fever (25, 28) may be due to survival of resting organisms in such tissues as the tonsil where host defenses may be ineffectual.

SUMMARY

When four-hour cultures of group A streptococci are incorporated in the center of agar disks and implanted in the peritoneal cavity of the rabbit, the organisms enter immediately the logarithmic phase of growth and continue to multiply at maximal rates for eight hours. Subsequently a plateau is reached and persists for five days. Large amounts of penicillin administered prior to or within five hours of implantation kill the bacteria promptly, but when given twelve hours or more after implantation, the drug fails to sterilize the foci. Although repeated administration decreases the bacterial count, some viable organisms persist.

The implications of these findings on the treatment and sequelae of streptococcal infection in man are discussed.

REFERENCES

1. Hobby, G. L., Meyer, K., and Chaffee, E., Activity of penicillin *in vitro.* Proc. Soc. Exper. Biol. & Med., 1942, **50**, 277.
2. Rammelkamp, C. H., and Keefer, C. S., Penicillin: Its antibacterial effect in whole blood and serum for the hemolytic streptococcus and staphylococcus aureus. J. Clin. Invest., 1943, **22**, 649.
3. Eriksen, K. R., Some studies on the lytic action of penicillin on staphylococci and pneumococci. Acta path. et microbiol. Scandinav., 1946, **23**, 221.
4. Eriksen, K. R., Studies on the action of penicillin on some rod-shaped, penicillinase-producing bacteria. Acta path. et microbiol. Scandinav., 1946, **23**, 489.
5. Parker, R. F., and Marsh, H. C., The action of penicillin on staphylococcus. J. Bact., 1946, **51**, 181.
6. Hobby, G. L., Meyer, K., and Chaffee, E., Observations on the mechanism of action of penicillin. Proc. Soc. Exper. Biol. & Med., 1942, **50**, 281.
7. Hobby, G. L., and Dawson, M. H., Effect of rate of growth of bacteria on action of penicillin. Proc. Soc. Exper. Biol. & Med., 1944, **56**, 181.

8. Lee, S. W., Foley, E. J., and Epstein, J. A., Mode of action of penicillin. I. Bacterial growth and penicillin activity—Staphylococcus aureus FDA. J. Bact., 1944, **48**, 393.
9. Todd, E. W., Bacteriolytic action of penicillin. Lancet, 1945, **1**, 74.
10. Jawetz, E., Dynamics of the action of penicillin in experimental animals; observations on mice. Arch. Int. Med., 1946, **77**, 1.
11. Eagle, H., Experimental approach to the problem of treatment failure with penicillin. I. Group A streptococcal infection in mice. Am. J. Med., 1952, **13**, 389.
12. Wood, W. B., Jr., Studies on the cellular immunology of acute bacterial infections. Harvey Lect., 1951–1952, **47**, 72.
13. Werner, C. A., Knight, V., and McDermott, W., Studies of microbial populations artificially localized *in vivo*. I. Multiplication of bacteria and distribution of drugs in agar loci. J. Clin. Invest., 1954, **33**, 742.
14. Fleming, A., Micro-methods of estimating penicillin in blood serum and other fluids. Am. J. Clin. Path., 1945, **15**, 1.
15. Spink, W. W., Rantz, L. A., Boisvert, P. J., and Coggeshall, H., Sulfadiazine and penicillin for hemolytic streptococcus infections of the upper respiratory tract; an evaluation in tonsillitis, nasopharyngitis, and scarlet fever. Arch. Int. Med., 1946, **77**, 260.
16. Plummer, N., Duerschner, D. R., Warren, H. D., Rogliano, F. T., and Sloan, R. A., Penicillin therapy in hemolytic streptococcic pharyngitis and tonsillitis. J.A.M.A., 1945, **127**, 369.
17. Smith, M. R., and Wood, W. B., Jr., Personal communication.
18. Rantz, L. A., The Prevention of Rheumatic Fever. Springfield, Ill., Charles C Thomas, 1952, p. 20.
19. Lemon, H. M., The nasal carrier of beta-hemolytic streptococci. New England J. Med., 1947, **237**, 988.
20. Rothbard, S., and Watson, R. F., Variation occurring in group A streptococci during human infection; progressive loss of M-substance correlated with increasing susceptibility to bacteriostasis. J. Exper. Med., 1948, **87**, 521.
21. Rantz, L. A., The hemolytic streptococci. Studies on the carrier state in the San Francisco area, with notes on the methods of isolation and serological classification of these organisms. J. Infect. Dis., 1941, **69**, 248.
22. Nelson, H. G., Studies on rheumatic fever. Observations on tonsillar carriers of hemolytic streptococci; the effect of tonsillectomy and the administration of penicillin on rheumatic and nonrheumatic fever patients. J. Infect. Dis., 1948, **83**, 138.
23. György, E., and Székely, Á., Absorption and effect of penicillin injected into the tonsils on the bacterial flora of tonsils. Acta oto-laryng., 1951, **39**, 62.
24. Lund, E., and Everberg, G., Preoperative penicillin therapy in tonsillectomy. Acta oto-laryng., 1952, **42**, 125.
25. Catanzaro, F. J., Stetson, C. A., Morris, A. J., Chamovitz, R., Rammelkamp, C. H., Jr., Stoltzer, B. L., and Perry, W. D., The role of the streptococcus in the pathogenesis of rheumatic fever. Am. J. Med., 1954, **17**, 749.
26. Rammelkamp, C. H., Jr., The natural history of streptococcal infections. Bull. New York Acad. Med., 1955, **31**, 103.
27. Massell, B. F., Sturgis, G. P., Knobloch, J. D., Streeper, R. B., Hall, T. N., and Norcross, P., Prevention of rheumatic fever by prompt penicillin therapy of hemolytic streptococcic respiratory infections. J.A.M.A., 1951, **146**, 1469.
28. Wannamaker, L. W., Rammelkamp, C. H., Jr., Denny, F. W., Brink, W. R., Houser, H. B., Hahn, E. O., and Dingle, J. H., Prophylaxis of acute rheumatic fever by treatment of the preceding streptococcal infection with various amounts of depot penicillin. Am. J. Med., 1951, **10**, 673.

ERRATUM

In the article "The Effect of Acute Arteriovenous Fistula on Renal Functions," by James G. Hilton, Donald M. Kanter, David R. Hays, Edward H. Bowen, James R. Golub, John H. Keating, and René Wégria, J. Clin. Invest., 1955, **34**, 732, left column, p. 735, line 18 ". . . occurred an increase in the sodium excretion while" should read ". . . occurred a decrease in the sodium excretion while"; line 20 ". . . upon closing the fistula (experiments 7 and 8) . . ." should read ". . . upon closing the fistula (experiments 4 and 8) . . ."

ESTIMATION OF THE SIZE OF THE PATENT DUCTUS ARTERIOSUS FROM HEMODYNAMIC DATA

By NOBLE O. FOWLER[1] AND EDGAR P. MANNIX

(From the Departments of Medicine and Surgery, State University of New York at New York City, and the Cardiac Laboratory, Brooklyn Hospital, N. Y.)

(Submitted for publication December 13, 1954; accepted March 30, 1955)

The effects of patent ductus arteriosus upon the circulation were studied by Eppinger, Burwell, and Gross (1). Later, Dexter, Haynes, Burwell, Eppinger, Sosman, and Evans (2) described preoperative and postoperative hemodynamic studies obtained by cardiac catheterization in a patient with patent ductus arteriosus. Since the presence of a patent ductus arteriosus can be detected by physical examination in more than 95 per cent of the patients having this condition (3), cardiac catheterization studies are needed as an aid to diagnosis only where the murmur is atypical, or when there is a possibility of an additional abnormality. When the single lumen cardiac catheter is used, it may be very difficult to distinguish between shunts of oxygenated blood into the upper right ventricle and shunts into the pulmonary artery (4). At times, aortography may be needed to settle the diagnosis unless the cardiac catheter is passed through the ductus arteriosus.

Hemodynamic data may, however, be of assistance in estimating the size of the patent ductus arteriosus (5). This information may be of value in contemplating interruption of the ductus in very young children, or in adults over the age of forty. In addition, the suggestion of a very large channel may warn the surgeon of possible technical difficulties in its ligation or division.

This paper describes in twelve patients a comparison between the diameter of the ductus as measured at operation and certain hemodynamic observations obtained preoperatively. In the preoperative study, the following values were determined: increase in oxygenation of pulmonary arterial over mixed venous blood; volume of left to right shunt through the ductus; the internal diameter of the ductus arteriosus calculated from an orifice formula (5).

[1] Present address: Department of Medicine, Emory University, Atlanta, Ga.

MATERIAL

The subjects studied were twelve patients in whom the diagnosis of patent ductus arteriosus was made clinically. Their ages were from four to thirty-nine years (Tables I and II). The diagnosis of uncomplicated patent ductus arteriosus was made on the basis of a continuous or quasi-continuous murmur, maximal in the pulmonary valve area, the absence of cyanosis, the presence of a normal electrocardiogram, and chest x-rays showing normal or increased pulmonary vascular markings with slight or moderate enlargement of the left atrium and left ventricle.

METHODS

Cardiac catheterization of the right heart and pulmonary artery was done in the usual manner (6), using a single lumen catheter. Multiple blood samples were obtained from the pulmonary artery, right ventricle, right atrium, and one or both venae cavae, and analyzed in the Van Slyke apparatus. Pulmonary arterial samples for flow calculations were obtained from the distal right pulmonary artery. Following this, the catheter was quickly withdrawn, and samples were taken in rapid succession from the main pulmonary artery, right ventricle, right atrium, and venae cavae. Simultaneous pulmonary and systemic arterial pressures were obtained immediately after simultaneous pulmonary and brachial arterial blood samples. Pressures were measured by means of Hathaway strain gages and oscillograph or Sanborn transducers and Poly-Viso electrocardiograph. Systemic arterial pressure was measured through an indwelling needle in the brachial or femoral artery. In the adults, oxygen consumption was measured for flow calculations by collection of two-minute samples of expired air in Douglas bags, measurement in a Tissot spirometer, and analysis in the Haldane apparatus or in a Scholander micro gas analyzer. Simultaneous pulmonary and systemic arterial samples were taken over a one-minute period midway during the collection of expired air. In the children, oxygen consumption was calculated on the basis of surface area; these children were either sedated with one mg. of morphine per ten pounds body weight and three mg. of sodium phenobarbital per Kg. body weight, or were anesthetized with a combination of rectal and intravenous Sodium Pentothal®. Pulmonary flow, effective pulmonary flow, and left-to-right shunt were calculated from the formulae of Bing, Vandam, and Gray (7). Left-to-right shunt through the

ductus was calculated as the difference between total pulmonary flow and effective pulmonary flow. Pulmonary venous blood oxygen content was assumed to be 95 per cent of capacity unless the systemic arterial oxygen content exceeded this value; in those instances, pulmonary venous blood oxygen content was taken to be the same as that for systemic arterial blood. The theoretical internal diameter of the patent ductus arteriosus was calculated in accordance with the formula of Gorlin and Gorlin (5). The external diameter of the ductus was measured at operation with a caliper, either directly or with the aid of a piece of silk suture. Since the ductus was ligated rather than divided, its internal diameter was not measured. The length of the ductus arteriosus was also estimated at operation, and although this value must be considered an approximation, the theoretical internal diameter of the patent ductus arteriosus was then recalculated using a formula for the flow of a fluid through a pipe (8), based on Poiseuille's law.

RESULTS

The results are summarized in Tables I and II. All patients save one (M. C.) had normal saturation with oxygen of peripheral arterial blood. All patients had an increase in oxygen content of pulmonary arterial blood above that in the right ventricle or that in mixed venous samples from the right atrium. In two patients (A. F. and V. M.) the increase in arterial oxygen content of pulmonary arterial over right ventricular blood was of borderline significance. A significant increase in oxygenation of right ventricular blood over that in the right atrium was found in three patients (J. M., G. P., and V. M.). This was presumably due to insufficiency of the pulmonary valve (4). In the two latter patients, these samples were taken only two minutes apart. Location of the catheter tip was confirmed by pressure tracings in each instance. Disappearance of all murmur after ligation of the ductus is considered to have eliminated the possibility of ventricular septal defect as a cause of oxygenation of right ventricular blood. Patient G. P. was catheterized postoperatively and no evidence of increased oxygenation of right ventricular or pulmonary arterial blood was found. The calculated shunt through the ductus ranged from 0.8 to 17.1 liters per minute. Nine patients had mild or moderate pulmonary hypertension; three had normal pulmonary arterial pressures. Measurement of pulmonary wedge or "capillary" pressure was made in five patients. The only elevation (17 mm. mercury) was seen in a

TABLE I

Hemodynamic data in 12 patients with patent ductus arteriosus

Patient Age Sex	Systemic artery oxygen content cc./100 cc. and % of cap.	Distal PA O₂ content cc./100 cc.	RV outflow O₂ content cc./100 cc.	Average rt. atrium O₂ content cc./100 cc. (Mixed venous)	Difference O₂ content cc./100 cc. and in % saturation* PA – MV		PA – RV		Oxygen consumption cc./min.	Systemic flow L./min.	Effective pulm. flow L./min.	Pulm. flow L./min.	Shunt, left-right L./min.
					cc.	%	cc.	%					
1. A. L., 34, F.	16.8	94	14.7	9.6	9.7	5.0	29	5.1	30	209	2.9	2.9	10.0	7.1
2. C. W., 23, F.	15.0	96	13.0	10.1	10.1	2.9	19	2.9	19	205	4.2	4.2	10.3	6.1
3. J. M., 14, F.	17.0	97	14.6	13.1	12.0	2.6	15	1.5	9	206	4.1	4.1	8.6	4.5
4. W. T., 21, M.	19.4	100	17.4	15.7	15.2	2.1	12	1.7	9	231	5.5	5.5	11.6	6.1
5. F. T., 39, F.	12.1	98	10.5	7.3	7.0	3.5	28	3.2	26	186	3.6	3.6	11.6	8.0
6. G. P., 30, F.	17.9	96	16.8	14.6	12.8	4.0	20	2.2	10	240	4.7	4.7	21.8	17.1
7. A. F., 26, F.	16.3	95	12.9	12.1	11.8	1.1	7	0.8	5	213	4.7	4.7	6.3	1.6
8. C. D., 6, F.	13.7	97	11.9	8.9	9.2	2.7	19	3.0	21	119	2.6	2.6	6.6	4.0
9. M. C., 5, M.	14.3	90	12.3	11.4	11.5	0.8	6	0.9	6	123	4.4	3.2	4.0	0.8
10. P. S., 8, F.	13.9	93	11.7	9.5	9.5	2.2	15	2.2	15	126	2.9	2.6	4.8	2.2
11. V. M., 6, M.	17.3	93	14.0	13.2	12.0	2.0	11	0.8	4	118	2.2	2.0	3.0	1.0
12. L. L., 4, F.	15.6	99	12.9	10.4	9.8	3.1	20	2.5	14	72	1.2	1.2	2.7	1.5

* PA = Pulmonary Artery,
RV = Right Ventricle,
MV = Mixed Venous (average of right atrial samples).

TABLE II

Pulmonary vascular pressures; calculated and observed diameter of ductus in 12 patients with patent ductus arteriosus

Patient Age Sex	PA pressure* sys./dias. mean	Pulm. wedge pressure*	Systemic arterial pressure* sys./dias. mean	Shunt, left-right *L./min.*	Calculated internal diameter *mm.* (Orifice formula)	Measured external diameter *mm.*	Measured length *mm.*	Calculated internal diameter (Pipe formula)
A. L., 34, F.	— 44	—	— 150	7.1	5.8	18	—	—
C. W., 23, F.	— 29	—	— 112	5.7	5.6	9	2.5	3.1
J. M., 14, F.	— 23	11	— —	4.5	—	8	6.0	—
W. T., 21, M.	40/12 21	—	— —	5.0	—	10	3.5	—
F. T., 39, F.	26/16 20	17	149/62 93	8.0	6.8	10	10	4.9
G. P., 30, F.	24/14 18	—	111/56 77	17.1	10.2	14	10	6.2
A. F., 26, F.	19/11 15	10	113/65 86	1.6	3.0	5.4	6	2.9
C. D., 6, F.	38/26 33	—	120/69 88	3.1	5.0	10	6	3.9
M. C., 5, M.	27/8 18	—	100/57 64	0.8	2.4	5.5	7	2.8
P. S., 8, F.	— 16	8	140/81 110	2.2	3.2	5.5	7	3.0
V. M., 6, M.	32/19 25	—	95/42 66	1.0	2.8	8.5	8	3.2
L. L., 4, F.	18/15 16	8	88/45 64	1.5	3.2	7	7	3.2

* Pressures are expressed in mm. mercury.

woman of 39 (F. T.) with a large ductus, a large heart, and the symptom of exertional dyspnea. In ten patients, calculation of the internal diameter of the ductus was made; the values obtained ranged from 2.4 to 10.2 mm. Observed external diameters were from 5.5 to 18 mm. Even with allowance for variation in the thickness of the walls of the ducti, the calculated values seemed in general too small.

The regression (9) of observed external diameter on calculated internal diameter showed a significant association, $p = 0.02$ (Figure 1). Regression of external diameter on increase in percentage of oxygen saturation of pulmonary arterial over mixed venous blood (average of right atrial samples) also showed significant association, $p = 0.01$ (Figure 2). Regression of external diameter of the ductus on left-to-right shunt showed also significant association, $p = < 0.02$ (Figure 3). An association was thus shown between the observed diameter of the ductus and each of the following: theoretical internal diameter; volume of shunt through the ductus; and increase in oxygenation of pulmonary arterial blood. The correlation was by no means linear, there being considerable scatter about the regression line. When the theoretical internal diameter of the ducti was recalculated with variation in length considered (8), a somewhat closer association was found (Figure 4), $p = 0.001$, than with the orifice formula (5).

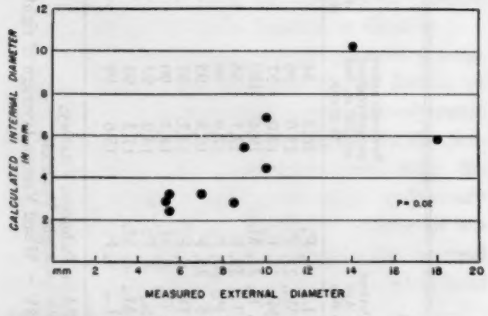


FIG. 1. RELATION OF MEASURED EXTERNAL DIAMETER TO CALCULATED INTERNAL DIAMETER IN TEN PATIENTS WITH PATENT DUCTUS ARTERIOSUS

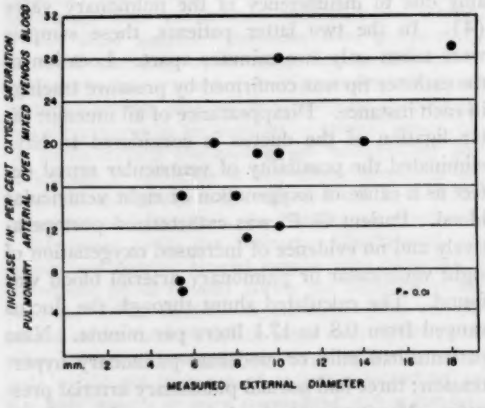


FIG. 2. RELATION BETWEEN MEASURED EXTERNAL DIAMETER AND INCREASE IN OXYGENATION OF PULMONARY ARTERIAL BLOOD IN TWELVE PATIENTS WITH PATENT DUCTUS ARTERIOSUS

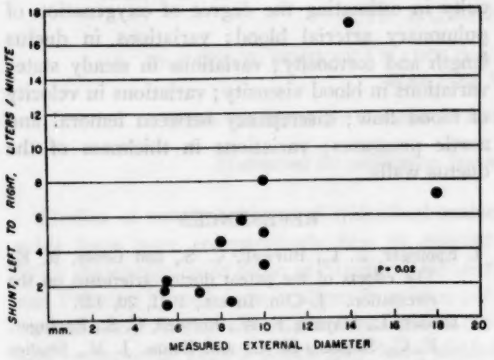


FIG. 3. RELATION BETWEEN MEASURED EXTERNAL DIAMETER AND LEFT-TO-RIGHT SHUNT IN TWELVE PATIENTS WITH PATENT DUCTUS ARTERIOSUS

DISCUSSION

In 1950, Taylor, Pollack, Burchell, Clagett, and Wood (10) published a study of the correlation between flow through the ductus and its external diameter measured at operation. No significant correlation was found. However, these workers did not calculate actual volume of flow, but rather expressed flow as a percentage of left ventricular output. Theoretically, one would expect the fraction of left ventricular output passing through the ductus to decrease with increase in body size and cardiac output, assuming the cross sectional area of the ductus to remain fixed, and a constant aorta-pulmonary artery pressure gradient. These

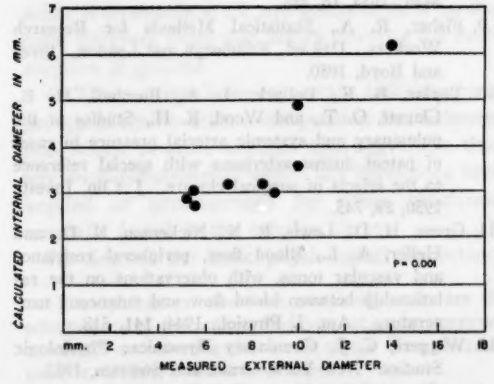


FIG. 4. RELATION BETWEEN MEASURED EXTERNAL DIAMETER AND CALCULATED INTERNAL DIAMETER IN NINE PATIENTS WITH PATENT DUCTUS ARTERIOSUS, USING THE POISEUILLE EQUATION

workers did find the most constant correlation between blood flow through the ductus arteriosus and the product of the square of the radius of the ductus and the square root of the aorta-pulmonary artery pressure gradient. This corresponds to the association we found between the calculated internal diameter and the observed actual diameter.

Failure to find linear correlation between actual size of the ductus and the hemodynamic data is not surprising. There are many sources of error in such a study. The determination of the degree of increased oxygenation of pulmonary arterial blood is quite difficult, because there is poor mixing of the two blood streams when samples are taken so near the site of entrance of oxygenated blood into the pulmonary artery. To reduce this source of error, blood samples for pulmonary flow calculation were taken from the distal right pulmonary artery rather than the main pulmonary artery. Eppinger, Burwell, and Gross (1) showed that there is equal mixing of oxygenated blood in both pulmonary arteries when there is an aortic-pulmonary arterial shunt into the main pulmonary artery of the dog. However, in humans, the patent ductus arteriosus may be on occasion inserted considerably to the left of the midline of the pulmonary bifurcation (1). In this event, a sample from either pulmonary artery would give an erroneous impression of the magnitude of the shunt from the aorta. The degree of oxygenation of pulmonary arterial blood is used in calculations of flow through the ductus, which value is used in turn in the calculation of the theoretical internal diameter of the ductus arteriosus. Any error in this value would cause a corresponding error in the two subsequent calculations. There is a possibility of error in the patients whose oxygen consumptions were assumed rather than measured. Variations in the steady state are a possible cause of error. In theory, one would expect the calculated flow through the ductus to be a better estimate of ductus size than the degree of increase in oxygenation of pulmonary arterial blood, since it includes the additional factor of the size of the subject. This was not seen in the data. The calculated internal diameter from the orifice formula (5) also failed to show a closer association with measured ductus size, although this determination includes the added element of pressure gradient on either side of the shunt.

This suggests the possibility, as seems likely, that the ductus does not behave as a perfect orifice, and that there is considerable energy loss within it due to its length and possible tortuosity. The inclusion of the observed length in the formula for calculation of ductus diameter seemed to improve the association somewhat, but it is difficult to draw a conclusion from so small a sample.

Still another possible cause of difficulty in the above comparisons, relative to the measurement of ductus size, is that of variation in the thickness of the walls of the ducti, since the internal diameters were not measured. Since fluid viscosity enters into the formula for flow through short pipes (8), variations in blood viscosity are a potential source of error. Green, Lewis, Nickerson, and Heller (11) have shown that the effective viscosity of the blood decreases with increasing rates of flow, since blood is a non-homogeneous liquid. Thus variations in velocity of flow may add to the error of these calculations. Because of wave summation, systolic pressures in the femoral artery may exceed those in the aorta by as much as 20 mm. mercury (12). Thus the use of femoral arterial pressure as an estimate of aortic arch pressure in children may contribute some error.

SUMMARY

In twelve patients having a patent ductus arteriosus and subjected to ligation of the ductus at operation, a comparison was made between the observed diameter of the ductus arteriosus and certain hemodynamic data obtained by preoperative cardiac catheterization. Significant association was found between actual ductus diameter and each of the following: degree of increase in oxygenation of pulmonary arterial blood; calculated volume of blood flow through the ductus; and calculated internal diameter of the ductus. The failure of the calculated internal diameter to be a more accurate estimate of true ductus diameter than either the volume of flow through the ductus or the degree of increase in oxygenation of pulmonary arterial blood suggests that the ductus does not behave as a perfect orifice. Among the sources of error in these comparisons are difficulty in estimating the degree of oxygenation of pulmonary arterial blood; variations in ductus length and tortuosity; variations in steady state; variations in blood viscosity; variations in velocity of blood flow; discrepancy between femoral and aortic pressures; variations in thickness of the ductus walls.

REFERENCES

1. Eppinger, E. C., Burwell, C. S., and Gross, R. E., The effects of the patent ductus arteriosus on the circulation. J. Clin. Invest., 1941, **20**, 127.
2. Dexter, L., Haynes, F. W., Burwell, C. S., Eppinger, E. C., Sosman, M. C., and Evans, J. M., Studies of congenital heart disease. III. Venous catheterization as a diagnostic aid in patent ductus arteriosus, tetralogy of Fallot, ventricular septal defect, and auricular septal defect. J. Clin. Invest., 1947, **26**, 561.
3. Gross, R. E., and Longino, L. A., The patent ductus arteriosus. Observations from 412 surgically treated cases. Circulation, 1951, **3**, 125.
4. Dammann, J. F., and Sell, C. G. R., Patent ductus arteriosus in the absence of a continuous murmur. Circulation, 1952, **6**, 110.
5. Gorlin, R., and Gorlin, S. G., Hydraulic formula for calculation of the area of the stenotic mitral valve, other cardiac valves, and central circulatory shunts. I. Am. Heart J., 1951, **41**, 1.
6. Cournand, A., and Ranges, H. A., Catheterization of the right auricle in man. Proc. Soc. Exper. Biol. & Med., 1941, **46**, 462.
7. Bing, R. J., Vandam, L. D., and Gray, F. D., Jr., Physiological studies in congenital heart disease, I, Procedures. Bull. Johns Hopkins Hosp., 1947, **80**, 107.
8. Mendlowitz, M., Intravascular resistance. Am. J. Med., 1954, **16**, 465.
9. Fisher, R. A., Statistical Methods for Research Workers. 11th ed., Edinburgh and London, Oliver and Boyd, 1950.
10. Taylor, B. E., Pollack, A. A., Burchell, H. B., Clagett, O. T., and Wood, E. H., Studies of the pulmonary and systemic arterial pressure in cases of patent ductus arteriosus with special reference to the effects of surgical closure. J. Clin. Invest., 1950, **29**, 745.
11. Green, H. D., Lewis, R. N., Nickerson, N. D., and Heller, A. L., Blood flow, peripheral resistance and vascular tonus, with observations on the relationship between blood flow and cutaneous temperature. Am. J. Physiol., 1944, **141**, 518.
12. Wiggers, C. J., Circulatory Dynamics; Physiologic Studies. New York, Grune and Stratton, 1952.

RENAL CLEARANCE OF EIGHTEEN INDIVIDUAL AMINO ACIDS IN HUMAN SUBJECTS[1]

By PAUL D. DOOLAN,[2] HAROLD A. HARPER, MAXINE E. HUTCHIN, AND WALTON W. SHREEVE[3]

(From the Metabolic Research Facility, U. S. Naval Hospital, Oakland, Calif.)

(Submitted for publication January 24, 1955; accepted April 6, 1955)

Studies of renal clearance of individual amino acids have been comparatively few in number. With the exception of those of Sheffner, Kirsner, and Palmer (1) such investigations have been performed solely on experimental animals and in certain respects the results which they have yielded have been conflicting. In part, the conflict has arisen because of the limited specificity of the analytical methods which the earlier investigators were forced to employ (2–7). Beyer and his associates (8–12), and Kamin and Handler (13), using the more specific methods of microbiological assay, were able to define these areas of conflict as well as to confirm and extend certain of the earlier findings. The most important findings of these previous investigations and the present state of knowledge on this subject might be summarized as follows:

1. In the fasting state the reabsorption of amino acids from the glomerular filtrate is almost complete.

2. When the filtered loads are increased, both the amounts of amino acid excreted and those which are reabsorbed are also increased.

3. Maximal reabsorptive capacities for amino acids, if attainable at all, are achieved by a mechanism different from that which governs the reabsorption of glucose.

4. There exists between certain individual and groups of amino acids a competition for reabsorption which is more obvious and aggressive than any such phenomenon which has heretofore been implied or demonstrated for other substances normally found in the tubular filtrate.

In view of the basic physiological importance of these findings and the interest demonstrated in recent years in various amino-acidurias, an investigation of the renal handling of individual amino acids by normal human subjects has been undertaken.

The proper utilization of a single amino acid depends on the simultaneous presence of a mixture of other amino acids in optimal concentrations; consequently, an attempt was made in these experiments to satisfy these requirements for amino acid utilization by infusing a solution which contained the eight amino acids shown by Rose (14) to be essential to the maintenance of nitrogen balance in normal human subjects. These "essential" amino acids were present in the same relative concentrations as suggested by Rose, and supplemental amounts of other amino acids were added to furnish additional nitrogen. During the infusion the clearances of 16 natural amino acids and 1 unnatural amino acid were measured. This approach to the problem requires the administration of a solution in which the individual amino acids are present in concentrations relative to one another that differ widely from those found in the plasma of fasting normal subjects. From this standpoint, the solution does not reproduce the usual circumstances under which the kidney deals with amino acids. To prepare a solution which would closely resemble normal plasma in the concentration of amino acids relative to one another is, however, not feasible because the sparingly soluble amino acids limit the amounts of the more soluble ones which could otherwise be used. It is recognized that an additional difficulty connected with the use of any mixture containing individual amino acids in different concentrations is that it makes comparison and interpretation of their renal clearances more difficult. This difficulty could be overcome by the infusion of a solution containing only one or possibly two amino acids but as has already been pointed out such an

[1] The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large.

[2] Lieutenant, Medical Corps, United States Navy.

[3] Present address: Brookhaven National Laboratory, Associated Universities, Inc., Upton, L. I., N. Y.

experiment may not be considered an entirely justifiable procedure from a physiological standpoint and at least with respect to lysine, arginine (11) and glycine (15), it has been shown that there is a quantitative difference in the renal excretion of these amino acids when infused alone, as compared to that which occurs when they are infused as a component of an amino acid mixture. In addition, it does not appear in any of the pathological conditions which have been studied that the renal reabsorptive defect is confined to a single amino acid (16). Infusions of single amino acids, although easier to work with, thus induce an element of artificiality which seriously limits any induction into clinical terms.

TABLE I

Composition of infusion mixture

Amino acid	Amino Acids Solution Merck* Lot W-111	Lot B-27+
	μM/ml.	
L-alanine	20.2	11.6
L-arginine	17.8	17.8
L-aspartic acid	0.65	0.90
L-cystine	0.004	0.09
L-glutamic acid	2.59	3.54
L-glutamine		1.51
Glycine	317	312
L-histidine	64.5	31.6
L-isoleucine	112	104
L-leucine	119	105
L-lysine	82.2	69.2
L-methionine	31.5	27.5
DL-methionine#	32.2	32.2
L-phenylalanine	55.8	31.5
DL-phenylalanine#	9.7	9.7
L-proline	30.4	23.5
L-serine	76.2	19.1
L-threonine	13.4	16.1
DL-threonine#	22.9	22.9
L-tryptophan	3.87	4.26
DL-tryptophan#	5.88	5.88
L-tyrosine	2.71	2.15
L-valine	59.0	55.6

* The supplies of amino acids solution were generously provided by Merck & Co., Inc.

+ The osmolarity of this solution was 978 milliosmols per liter.

Supplements to original amino acid solution as supplied by Merck.

METHODS

Four clearance studies were performed in three normal individuals. Two were young, healthy Navy personnel—WB (wt. 150 lb., ht. 73 in., age 22) and DNW (wt. 190 lb., ht. 72.5 in., age 21). The other subject, WL (wt. 145 lb., ht. 67 in., age 60) had his left leg amputated years earlier. He originally volunteered to serve as a control for similar studies being performed on a 50-year-old nephrotic and since he was normal in all other respects, he is included in the present report.

Urine collection periods of 15 to 20 min. were used. The bladder was rinsed with 15 ml. of saline followed by 15 ml. of air. In the first three clearances, urine flow rates during amino acid infusion of 3 to 4 ml. per min. were used because it was felt that excessive dilution might interfere with the amino acid analyses. Subsequently, it was found that the analyses were reproducible within a wide dilution range so that in later experiments, larger urine flows were maintained (6 to 12 ml. per min.). The glomerular filtration rate was measured by means of inulin clearance (17). The values given are uncorrected for surface area. After three control periods, 100 ml. of an amino acid mixture (Table I) were injected as a priming dose. A constant infusion of this solution was then started and after a 15-minute equilibration period, three more clearance periods were performed. In the first two studies the inulin and amino acid infusions were given at rates of 3 to 4 ml. per min. by means of a gravity drip. In the last two experiments, they were given at rates of 4.4 (WB) and 4.1 (DNW) ml. per min. by means of a constant infusion pump (Bowman).

The blood levels of the amino acids remained reasonably constant throughout the period of study but the variation in level obtained for any particular amino acid among different subjects was rather unpredictable. Since the individual amino acids are present in the infusion mixture in widely different concentrations (Table I) and are substances which are rapidly metabolized, the relative constancy in serum concentrations which were obtained was considered very satisfactory. Venous blood samples were taken at the midpoint of the control periods and at the beginning and end of each period during the infusion of both inulin and the amino acid solutions. No attempt at correction for renal dead space was made.

Seventeen natural amino acids were measured by microbiological assay using methods previously described (18). One unnatural amino acid, D-methionine, was measured by difference, using *Lactobacillus fermenti* to measure DL-methionine and *Leuconostoc mesenteroides* to measure L-methionine. Since the infusion mixture contains only free amino acids, only these forms were measured. No attempt was made to determine combined amino acids by comparison of values obtained before and

TABLE II

Plasma concentration, filtered load and excretion values of the individual amino acids during a typical experiment on subject WB

Amino acids	Plasma concentration					Filtered load				Excretion			
	Fasting	Period after loading*				Fasting (112)†	Period after loading			Fasting	Period after loading		
			1	2	3		1(121)†	2(137)†	3(125)†		1	2	3
	μM./100 ml.					μM./min.				μM./min.			
L-alanine	28.1	41.6	41.6	39.3	39.3	31.5	50.4	55.4	49.2	0.28	0.08	0.15	0.09
L-arginine	16.1	28.8	28.8	28.8	27.0	18.1	35.1	39.7	34.5	nil	0.38	0.45	0.54
L-cystine	9.0	8.9	7.7	8.1	7.6	10.1	10.1	10.8	9.8	0.20	0.44	0.41	0.41
L-glutamine	52.7	56.9	56.2	56.2	60.3	58.9	68.5	76.7	72.6	0.58	1.13	0.98	0.84
Glycine	26.7	150.6	117.3	120.0	125.3	29.9	162.6	162.6	153.3	1.07	52.3	49.9	44.4
L-histidine	8.8	20.3	19.0	17.1	16.1	9.9	23.8	24.8	20.7	0.55	3.87	3.87	3.55
L-isoleucine	9.2	50.4	48.1	48.1	50.4	10.2	59.6	65.7	61.8	nil	0.21	0.23	0.21
L-leucine	14.5	71.8	70.2	73.3	74.8	16.3	86.3	98.5	92.4	0.04	0.38	0.30	0.29
L-lysine	16.4	56.9	53.4	50.7	45.2	18.4	66.4	71.2	60.3	0.03	0.49	0.53	0.47
L-methionine	3.36	16.6	17.2	18.7	18.8	0.38	20.3	24.6	23.4	0.013	0.89	0.99	0.94
D-methionine	nil	17.5	17.5	16.1	22.8	nil	21.1	23.0	24.6	nil	8.7	11.1	10.7
L-phenylalanine	9.4	20.9	19.7	19.4	18.5	10.6	24.7	26.9	23.7	0.07	0.36	0.45	0.42
L-proline	14.8	29.6	27.8	26.1	27.0	16.5	34.7	37.0	33.1	0.04	0.12	0.11	0.10
L-serine	14.3	22.9	21.9	23.8	23.8	16.0	27.2	31.3	29.8	0.21	2.98	3.26	3.21
L-threonine	12.6	22.7	22.7	21.0	21.9	14.1	27.5	30.1	27.0	0.08	1.41	1.59	1.44
L-tryptophan	5.9	11.7	12.0	12.2	12.4	6.6	14.2	16.5	15.3	0.10	0.35	0.34	0.34
L-tyrosine	4.42	7.2	6.6	6.6	6.6	5.0	8.3	9.1	8.3	0.10	0.11	0.13	0.11
L-valine	24.8	59.8	63.3	65.0	67.5	27.8	74.4	88.0	82.9	0.03	0.19	0.20	0.21

* Blood amino acid concentrations were determined at the beginning and end of each collection period.

† Glomerular filtration rates.

after hydrolysis. The overall precision of microbiological assays is ± 10 per cent. Alanine and arginine are difficult to measure with the same degree of reproducibility obtainable with the other amino acids. Aspartic and glutamic acids were also measured during these studies but because of the extremely small amounts normally present in the urine and plasma and since the infusion mixture had been rendered virtually free of both, the changes observed were considered of dubious significance and are not included. The results for cysteine/cystine have been reported arbitrarily in terms of cystine.

RESULTS

Endogenous clearance and response to load

In Table II the results of a typical experiment on subject WB are given. It will be noted that the fasting excretion or endogenous clearance of each amino acid is extremely low, indicating almost complete tubular reabsorption. With glycine and histidine somewhat less of the filtered loads, 96 and 94 per cent, respectively, were reabsorbed. In an earlier study in which the basal

TABLE III

Endogenous clearances of the individual amino acids

Amino acid	Subject				Mean
	WL	WB	WB	DNW	
	ml./min.				
L-valine	0.19	0.14	0.04	0.11	0.12
L-methionine	nil	0.40	nil	0.35	0.19
L-proline	0.13	0.29	0.32	0.13	0.22
L-lysine	0.28	0.21	0.15	0.24	0.22
L-leucine	0.21	0.26	0.23	0.36	0.27
L-isoleucine	0.44	nil	0.06	0.87	0.34
L-alanine	0.27	1.0	0.48	0.26	0.50
L-glutamine	0.56	1.1	0.63	0.38	0.67
L-arginine	2.45	nil	0.13	0.48	0.77
L-threonine	0.50	0.60	1.43	0.61	0.79
L-phenylalanine	0.24	0.71	1.26	1.00	0.80
L-tryptophan	1.18	1.67	1.97	0.67	1.37
L-serine	1.20	1.47	2.07	1.25	1.50
L-tyrosine	1.29	2.40	1.65	1.21	1.65
L-cystine	1.96	2.20	2.21	2.23	2.15
Glycine	6.3	4.0	5.4	4.8	5.12
L-histidine	6.1	6.3	6.5	4.6	6.38

excretion of amino acids was measured in 22 normal male subjects it was found that glycine, histidine and glutamine were excreted in the largest amounts (18, 19). In Table III the endogenous clearances for the individual amino acids in each of the present experiments and the average values are listed. It will be noted that histidine and glycine are also cleared in the largest amounts but that the clearance of histidine exceeds that of glycine. In the case of glutamine which, as mentioned, has a large basal excretion it will be noted that the endogenous clearance is quite small.

When the blood levels and filtered loads were increased there were increases in the amounts excreted. The increase in the amount excreted varied among individual amino acids and in this particular study tyrosine showed no significant increase. Other experiments have shown that tyrosine shares in this typical response. In any event the total amount of amino acid excreted represents only a very small part of that administered (< 4 per cent). The most significant event, quantitatively speaking, was an increase in the amount of amino acid reabsorbed per minute. Despite definite and in some instances marked elevations in serum concentrations, only five natural amino acids were ever excreted at a rate greater than 0.5 mg. per min., glycine > histidine > serine > threonine and on one occasion, lysine.

Comparison of individual clearances

In Table IV are listed for each subject the mean elevations in plasma concentrations obtained after amino acid loading, expressed as multiples of the fasting values, together with the grand mean for all subjects. The general pattern is as expected in that those amino acids present in the largest

TABLE IV

Elevations in concentration of the amino acids in the plasma expressed as multiples of the fasting values

Amino acid	Subject				Grand mean
	WL	WB	WB	DW	
	Mean				
L-cystine	0.99	0.89	0.86	0.93	0.92
L-glutamine	1.10	1.08	1.10	1.17	1.11
L-tyrosine	1.76	1.52	1.35	1.28	1.48
L-alanine	1.51	1.44	1.97	1.60	1.63
L-threonine	2.14	1.75	1.73	1.43	1.76
L-serine	1.62	1.61	1.76	2.04	1.76
L-tryptophan	2.06	2.04	1.89	1.62	1.90
L-proline	2.40	1.85	2.22	1.40	1.97
L-arginine	1.61	1.76	2.44	2.32	2.03
L-histidine	2.50	2.05	2.70	2.09	2.34
L-valine	2.53	2.58	2.96	1.93	2.50
L-phenylalanine	4.15	2.09	2.98	1.88	2.78
L-lysine	5.00	3.14	2.89	2.37	3.35
L-isoleucine	5.04	5.33	4.62	3.75	4.69
L-leucine	5.26	4.98	4.73	4.09	4.77
Glycine	7.33	4.70	6.49	5.29	5.95
L-methionine*	17.5	5.31	10.9	5.58	9.82

* The values found for methionine are of doubtful significance because of the extremely small amounts normally present in the plasma.

TABLE V

Clearances of amino acids during an infusion of a mixture of amino acids

Amino acid	Subject WL	WB Mean	WB	DNW	Grand mean
	ml./min.				
L-proline	0.58	0.41	0.48	0.54	0.50
L-valine	0.70	0.31	0.47	0.57	0.51
L-isoleucine	0.62	0.44	0.56	1.03	0.66
L-alanine	1.76	0.26	0.77	0.29	0.77
L-leucine	0.85	0.45	0.97	1.09	0.84
L-arginine	2.02	1.62	1.36	1.08	1.52
L-glutamine	2.82	1.73	2.83	1.89	2.32
L-phenylalanine	2.10	2.09	2.71	2.77	2.42
L-tyrosine	4.00	1.73	3.19	1.04	2.49
L-lysine	2.25	0.97	4.35	3.70	2.82
L-tryptophan	3.78	2.97	4.63	1.97	3.34
L-cystine	4.03	5.21	7.39	5.81	5.61
L-methionine	4.60	5.27	6.16	9.31	6.34
L-threonine	14.7	6.7	16.7	7.1	11.3
L-serine	21.7	13.7	34.8	14.3	21.1
L-histidine	32.2	20.8	39.4	28.7	30.3
D-methionine	49	57	47	56	52
Glycine	54	39	62	63	55

amounts in the mixture are also those which exhibited greatest elevations of concentration in the plasma. In Table V the mean clearance achieved during the infusion of amino acids for each of the four experiments, as well as the grand mean, are listed. Glycine, D-methionine, histidine, serine, and threonine have the largest clearances under load. However, the amount of plasma cleared of a given amino acid was not predictable merely from the rise in the concentration of this amino acid which was produced in the plasma as a result of the infusion. This implies that there is a considerable variation among different amino acids in the selectivity with which these substances are reabsorbed by the renal tubules.

Changes in glomerular filtration rate during amino acid infusion

In two of the four experiments, subjects WB and DNW, a moderate increase in glomerular filtration rate was observed. In Table II the values obtained in subject WB are given. In the other subject (DNW) in whom an increase in GFR was also observed the values obtained during the control period were 145 and 155, and during the amino acid infusion periods, 168, 175, and 168 ml. per min. Since there was a considerable variation from subject to subject in the plasma concentrations produced by the amino acid infusion, it is not possible to state from the present data to what extent the alterations in filtration rate influenced the amino acid clearances. Additional studies are currently in progress in an attempt to define more exactly the effect of amino acid infusions on renal hemodynamics.

Clearances of D- and L-methionine

The clearances of the D- and L-forms of methionine were specifically compared (Table V) since an earlier study in this laboratory had shown a

TABLE VI

Comparison of the clearances of the D- and L- forms of methionine and the effect of water diuresis (Subject, WB)

	PERIODS				
	4	5	6	7	8
	ml./min.				
Urine flow	5.0	5.0	5.3	13.3	22.9
Clearance					
D-methionine	49.	50.	55.	79.	77.
L-methionine	4.6	4.0	4.3	3.0	2.8

greater excretion of the D-form (20). It was noted in the present study that the clearances of the D-form were substantially higher (47 to 57 ml. per min.) than those of the L-form (4.6 to 9.3 ml. per min.).

In the above studies the effect of urine flow on the reabsorption of the two forms was not studied. In subject WB, a water load was administered at a time when a similar clearance study was being performed after nine days of intravenous ACTH therapy which, incidentally, did not alter the reabsorption of amino acids in normal subjects (21). The results are reproduced in Table VI, where it will be noted that a significant increase in clearance of the D-form occurred with the onset of diuresis. These findings are similar to those which Crampton and Smyth demonstrated in the cat and which led them to conclude that the unnatural enantiomorphs of amino acids were reabsorbed passively while the natural forms were reabsorbed, at least in part, by an active mechanism (22). Dent, Senior, and Walshe have recently

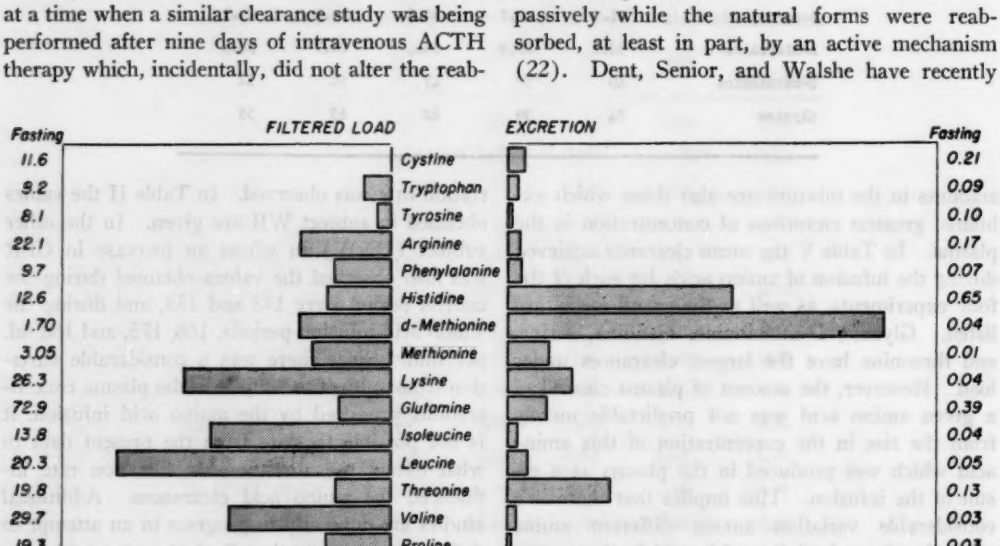


FIGURE 1

shown that in normal subjects the excretion of the D-form of cystine is much greater than that of the L-form (23).

DISCUSSION

In the fasting state the reabsorption of amino acids from the glomerular filtrate was almost complete. However, during a continuous infusion of an amino acid solution so that both the plasma level and the filtered loads are significantly increased, there was an increase in both the amounts reabsorbed and those excreted. The increase in the amounts reabsorbed far outweighed that excreted so that during the entire infusion period less than 4 per cent of the administered amino acids was excreted.

Because of this pattern of renal excretion and because of the differences in the degree to which the concentration of each amino acid was increased in the plasma, it is not possible with these data to derive a valid mathematical expression for the manner in which the individual amino acids were cleared relative to one another. The endogenous clearance, per cent excretion and/or per cent reabsorption are of dubious value as reference points.

Although the individual amino acids still cannot be compared, in the strict sense, an attempt has been made in Figure 1 to present the accumulated data in such a manner that certain general concepts concerning renal mechanisms for the excretion of amino acids can be gained. Figure 1 was prepared as follows: The columns headed "fasting" represent the mean value of the four subjects for the filtered load or excretion, respectively, of each amino acid in the fasting state. Similar mean values were then calculated for all of the experiments performed after infusion of the amino acid mixture, and horizontal bars used to represent the *differences* between the first (fasting) and second (load) set of mean values. It should be noted that the scale designating the amounts of amino acids excreted is 10 times larger than that referring to the amounts filtered. The individual amino acids are listed in Figure 1 in order of increasing molecular weight but no relationship of molecular weight *per se* to the absolute or relative amounts of amino acid excreted was apparent.

Despite significant and in some instances striking increases in the filtered loads of leucine, isoleucine, valine, alanine, arginine, proline, lysine, L-methionine, and phenylalanine, the amounts excreted remained extremely low indicating that they were reabsorbed by a mechanism or mechanisms which were still highly efficient. The data would also imply that whenever these amino acids are excreted in large amounts, it is more likely due to an impairment of the normal reabsorption mechanism than a saturation phenomenon. Chemically, this group of amino acids is heterogeneous, being composed of neutral acids of aliphatic, aromatic, heterocyclic and sulfur-containing types, as well as two basic amino acids.

The amounts of histidine, serine, threonine, and glycine excreted did rise significantly under load, indicating less complete reabsorption. These are the amino acids for which it might be anticipated that maximal reabsorptive capacities could be demonstrated and such work is currently in progress. Chemically, the group consists of three neutral monamino monocarboxylic aliphatic acids and one basic heterocyclic acid.

Earlier work in this laboratory, as well as that of other investigators, has shown that histidine (17, 18, 24–26) and glycine (17, 18, 26) are among those amino acids normally excreted in the largest amounts. It also should be mentioned that Eckhardt and Davidson found a proportionately larger excretion of threonine and histidine following intravenous infusion of the same amino acid solution used in this study than of any other of the amino acids studied (25). Kamin and Handler have called attention to the fact that the excretion of histidine and threonine are most frequently increased by the infusion of another single amino acid and postulated this as a possible explanation for the large excretions of these two amino acids in a variety of normal and abnormal states (13).

Interpretation of the increases in excretion of glutamine, tyrosine and tryptophan is rendered difficult by the small increases in the amounts filtered during the load period. Glutamine may occupy a position intermediate between the first two groups of amino acids.

The increase in excretion of cystine in the absence of any increase in filtered load is noteworthy. Pitts (5, 6), Beyer and his co-workers (9), and

Kamin and Handler (13) have all presented evidence that in the experimental animal there exists a competition between individual amino acids for reabsorption. Corroborating evidence is available in the present studies in the case of cystine. There was virtually no cystine in the mixture of amino acids which was infused and there was no significant change in the concentration of cystine in the plasma in any of the four experiments. Despite this, the average clearance of cystine during the infusion periods was 5.6 ml. per min. whereas the endogenous clearance in the four experiments were 2.2, 2.2, 2.2, and 2.0 ml. per min. This indicates that one or more amino acids were reabsorbed at the expense of cystine. The results could not have been due to changes in urine flow for no correlation has been demonstrated in these experiments between urine flow and cystine clearance. Although it is conceivable that something in addition to cystine was being measured in the urine, we have no evidence for such an explanation and seriously doubt that its additive effect would be great enough to account for the results obtained. In earlier work in this laboratory it was found that homocystine, cystathionine, and homolanthionine failed to support the growth of the test organism and the activity of glutathione was always less than 20 per cent of its cysteine content (27). To what extent, if any, other amino acids were involved in such a competitive phenomenon cannot be stated on the basis of the present data, but it is a reasonable assumption that the phenomenon is not limited to cystine. The finding was actually somewhat unexpected for, in earlier studies wherein a single injection of different amino acid mixtures was administered and their excretion measured in a three-hour specimen, no significant change in cystine excretion was observed (19). However, in those studies lower concentrations of amino acids in the plasma were obtained and the urine collection period was prolonged beyond the point where acute changes would be detected. It should also be mentioned that Dent, Senior, and Walshe were unable to demonstrate in a normal subject any competition for reabsorption between cystine and lysine, ornithine, and arginine (23).

The infusion of this particular type of amino acid solution, a solution which is both hypertonic and acidic, was associated on two occasions with an increase in glomerular filtration rate. Whether this is a pharmacological effect attributable to the solution or a physiological response to the elevation in the concentrations of amino acids in the plasma remains to be determined. Other factors, such as alterations in the body fluid compartments may possibly have been operative. Pitts infused single amino acids or combinations of amino acids into dogs and observed an initial increase and subsequent decrease in glomerular filtration rate (5, 6). On the other hand, Wright (10) and Russo (12) noted no significant effect on either filtration rate or renal blood flow with the infusion of single amino acids or combinations thereof, or when an acid hydrolysate of casein was infused. To what extent the increase in filtration rate influenced the results obtained cannot be stated. It is interesting, however, to note that the increased excretion of histidine which is found in pregnancy has been ascribed largely to the normal increase in glomerular filtration rate which occurs during most of the period of pregnancy (28).

SUMMARY

1. The renal clearances of 18 individual amino acids have been studied in three normal human subjects by the simultaneous measurement of inulin and amino acid excretion during periods of fasting as well as during the infusion of an amino acid mixture.

2. Excretion during fasting, *i.e.*, the endogenous clearance of each amino acid measured, was extremely low, indicating that the reabsorption of amino acids from the glomerular filtrate was almost complete at the plasma levels which prevail in the fasting state.

3. During a continuous infusion of an amino acid solution, there was an increase in the amounts of amino acids reabsorbed as well as those excreted. However, even under these conditions of loading, less than 4 per cent of the administered amino acids was excreted.

4. The excretion of isoleucine, leucine, valine, proline, alanine, methionine, arginine, phenylalanine, and lysine remained small despite significant and in some instances striking elevations in plasma concentrations. Histidine, serine, glycine, and threonine were excreted in larger amounts when the plasma levels were increased.

5. Evidence for competition between individual amino acids for tubular reabsorption was obtained in the case of cystine.

6. The clearance of the unnatural D-isomer of methionine was much higher than that of the natural, L-form. In the presence of a diuresis induced by a water load, a significant increase in clearance of the D-form occurred while that of the L-form decreased. This is taken as evidence for passive reabsorption of the D-form.

ACKNOWLEDGMENT

The constructive comments and advice of Drs. Thomas J. Kennedy, Jr., and Robert W. Berliner are gratefully acknowledged.

REFERENCES

1. Sheffner, A. L., Kirsner, J. B., and Palmer, W. L., Studies on amino acid excretion in man. I. Amino acids in urine. J. Biol. Chem., 1948, **175**, 107.
2. Doty, J. R., Reabsorption of certain amino acids and derivatives by the kidney tubules. Proc. Soc. Exper. Biol. & Med., 1941, **46**, 129.
3. Goettsch, E., Lyttle, J. D., Grim, W. M., and Dunbar, P., The renal amino acid clearance in the normal dog. Am. J. Physiol., 1944, **140**, 688.
4. Eaton, A. G., Ferguson, F. P., and Byer, F. T., The renal reabsorption of amino acids in dogs: Valine, leucine, and isoleucine. Am. J. Physiol., 1946, **145**, 491.
5. Pitts, R. F., A renal reabsorptive mechanism in the dog common to glycin and creatine. Am. J. Physiol., 1943, **140**, 156.
6. Pitts, R. F., A comparison of the renal reabsorptive processes for several amino acids. Am. J. Physiol., 1944, **140**, 535.
7. Ferguson, F. P., Eaton, A. G., and Ashman, J. S., Renal reabsorption of methionine in normal dogs. Proc. Soc. Exper. Biol. & Med., 1947, **66**, 582.
8. Beyer, K. H., Wright, L. D., Russo, H. F., Skeggs, H. R., and Patch, E. A., The renal clearance of essential amino acids: Tryptophane, leucine, isoleucine, and valine. Am. J. Physiol., 1946, **146**, 330.
9. Beyer, K. H., Wright, L. D., Skeggs, H. R., Russo, H. F., and Shaner, G. A., Renal clearance of essential amino acids: Their competition for reabsorption by the renal tubules. Am. J. Physiol., 1947, **151**, 202.
10. Wright, L. D., Russo, H. F., Skeggs, H. R., Patch, E. A., and Beyer, K. H., The renal clearance of essential amino acids: Arginine, histidine, lysine, and methionine. Am. J. Physiol., 1947, **149**, 130.
11. Wright, L. D., Renal clearances of essential amino acids. Tr. New York Acad. Sc., 1948, Ser. II, **10**, 271.
12. Russo, H. F., Wright, L. D., Skeggs, H. R., Tillson, E. K., and Beyer, K. H., Renal clearance of essential amino acids: Threonine and phenylalanine. Proc. Soc. Exper. Biol. & Med., 1947, **65**, 215.
13. Kamin, H., and Handler, P., Effect of infusion of single amino acids upon excretion of other amino acids. Am. J. Physiol., 1951, **164**, 654.
14. Rose, W. C., Amino acid requirements of man. Federation Proc., 1949, **8**, 546.
15. Doolan, P. D., Hutchin, M. E., and Harper, H. A., Unpublished observations.
16. Jonxis, J. H. P., and Huisman, T. H. J., Amino aciduria and ascorbic acid deficiency. Pediatrics, 1954, **14**, 238.
17. Roe, J. H., Epstein, J. H., and Goldstein, N. P., A photometric method for the determination of inulin in plasma and urine. J. Biol. Chem., 1949, **178**, 839.
18. Harper, H. A., Hutchin, M. E., and Kimmel, J. R., Concentrations of nineteen amino acids in plasma and urine of fasting normal males. Proc. Soc. Exper. Biol. & Med., 1952, **80**, 768.
19. Harper, H. A., Hutchin, M. E., Doolan, P. D., and Shreeve, W. W., Unpublished observations.
20. Kinsell, L. W., Harper, H. A., Barton, H. C., Hutchin, M. E., and Hess, J. R., Studies in methionine and sulfur metabolism. I. The fate of intravenously administered methionine in normal individuals and in patients with liver damage. J. Clin. Invest., 1948, **27**, 677.
21. Shreeve, W. W., Hutchin, M. E., Harper, H. A., Doolan, P. D., and Miller, C. D., Excretion of amino acids in nephrosis. Proc. Soc. Exper. Biol. & Med., 1955, **88**, 510.
22. Crampton, R. F., and Smyth, D. H., The excretion of the enantiomorphs of amino-acids. J. Physiol., 1953, **122**, 1.
23. Dent, C. E., Senior, B., and Walshe, J. M., The pathogenesis of cystinuria. II. Polarographic studies of the metabolism of sulphur-containing amino-acids. J. Clin. Invest., 1954, **33**, 1216.
24. Hier, S. W., Urinary excretion of individual amino acids on normal and low protein diets. Tr. New York Acad. Sc., 1948, Ser. II, **10**, 280.
25. Eckhardt, R. D., and Davidson, C. S., Urinary excretion of amino acids following the rapid injection of a solution of amino acids in man. J. Clin. Invest., 1948, **27**, 727.
26. Stein, W. H., A chromatographic investigation of the amino acid constituents of normal urine. J. Biol. Chem., 1953, **201**, 45.
27. Hutchin, M. E., Harper, H. A., Margen, S., and Kinsell, L. W., Microbiological determination of cystine, cysteine, and glutathione in plasma. J. Biol. Chem., 1950, **185**, 839.
28. Page, E. W., Glendening, M. B., Dignam, W., and Harper, H. A., The causes of histidinuria in normal pregnancy. Am. J. Obst. & Gynec., 1954, **68**, 110.

STUDIES ON KETONE METABOLISM IN MAN. I. A METHOD FOR THE QUANTITATIVE ESTIMATION OF SPLANCHNIC KETONE PRODUCTION [1, 2]

By EMILE E. WERK, JR.,[3] HARRY T. McPHERSON,[4] LADD W. HAMRICK, JR.,[5] JACK D. MYERS, AND FRANK L. ENGEL

(From the Department of Medicine, Duke University, Durham, N. C.)

(Submitted for publication January 10, 1955; accepted April 6, 1955)

The phenomenon of ketosis has interested investigators and clinicians for many years. With the recent rapid strides in the understanding of fat metabolism and the central role of acetyl-CoA in intermediary metabolism (1–5), this subject has taken on added interest. Although it has long been recognized that the liver is the chief site of origin of the ketone bodies, the basis for this has only recently been clarified. Apparently, most tissues are capable of forming acetoacetyl-CoA during the normal course of fatty acid catabolism or by condensation of two molecules of acetyl-CoA, but only the liver both possesses an enzyme, deacylase, which removes CoA from the molecule to release free acetoacetate, and at the same time lacks the acetoacetate activating enzyme which would result in acetoacetate's re-entering the metabolic cycle as acetoacetyl-CoA. Other tissues have some deacylase activity, but their acetoacetate activating enzyme system is so active that little or no free acetoacetate ever escapes from the cell (4). Ketonemia thus represents the balance between the rate of ketone production by the liver, utilization by extra hepatic tissues and excretion by the kidneys. Hepatic ketone production in turn is a function of the relative rates at which acetyl-CoA and acetoacetyl-CoA are formed, as from fat catabolism, and amino acids, and the rate at which acetyl-CoA can be disposed of by oxidation *via* the Krebs cycle or by reduction and synthesis to fat. Whenever the rate of acetyl-CoA formation exceeds that of disposal, two molecules of acetyl-CoA may condense to form acetoacetyl-CoA and this, as well as acetoacetyl-CoA derived directly from fat catabolism, may be released into the general circulation. The two pathways of acetate utilization (oxidation and fat synthesis) are strongly dependent on a relatively high rate of carbohydrate utilization, a fact which accounts for the well known occurrence of ketosis during such states of carbohydrate deprivation as occur during starvation and in diabetes mellitus (5–7).

It is thus apparent that ketone body production by the liver is a reflection of the balance of various forces in the metabolism of acetyl-CoA and hence it might be anticipated that any technique which enabled one to estimate hepatic ketone body production quantitatively should throw some light on the metabolism of this key metabolite in man. This has been made possible by the development of a sensitive method for the analysis of ketone bodies and the availability of a technique for estimating splanchnic blood flow by hepatic venous catheterization and bromsulphalein infusion. Moreover, the introduction by Margen, Michaels, Boling, and Kinsell (8) of sodium octanoate, an eight carbon fatty acid, for intravenous infusion has presented the opportunity of studying changes in splanchnic ketone production after loading with an exogenous precursor for ketone synthesis.

The present study represents the application of these techniques to the estimation of splanchnic ketone balances before and after octanoate infusion in essentially normal humans. It serves as a basis for future studies in this laboratory on the influence of hormones and of various pathological states on ketone metabolism in man.

METHODS

Twenty-one mildly ill hospital patients between the ages of twenty-three and sixty-two, without known

[1] Presented at the 45th Annual Meeting, the American Society for Clinical Investigation, Atlantic City, N. J., May 4, 1953, (J. Clin. Invest., 1953, 32, 610).

[2] Supported in part by research grants from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, Public Health Service (A142) and from Merck and Company, Rahway, N. J.

[3] American Cancer Society Fellow, 1953–1954. Present address: Veterans Administration Hospital, 3200 Vine Street, Cincinnati, Ohio.

[4] American College of Physicians Fellow, 1954–1955.

[5] American College of Physicians Fellow, 1952–1953.

metabolic abnormality, fever, or hepatic or renal disease, were used as subjects. Two patients (J. T. and D. B., Table I) were studied in the afternoon six hours post cibum; all others were studied in the morning after an overnight fast. The patients had been eating the regular hospital diet, with the exception of those with a peptic ulcer who had been consuming a liberal ulcer diet. All had been eating well, without recent weight loss, nausea or vomiting.

The patients were sedated with 90 to 120 mg. of phenobarbital given orally one to two hours before the studies. To minimize anxiety during the period of study, inlying femoral arterial and forearm venous needles and the hepatic venous catheter were inserted early in the procedure, using local procaine anesthesia, with no added epinephrine. An intravenous infusion of bromsulphalein was started, to be maintained, using a constant infusion pump, throughout the entire time of the study. After the needles and catheter were in place the patients were allowed to rest quietly for ten to fifteen minutes before the painless procedure of sampling was initiated.

A control study was carried out on each subject during which femoral arterial and hepatic venous bloods were each sampled four times, *i.e.*, every five minutes over a fifteen-minute period, for the subsequent determination of bromsulphalein, glucose and total ketone concentrations. A 500 ml. infusion of a 1.5 per cent solution of sodium octanoate was then started intravenously through a needle previously inserted and administered at a constant rate over approximately a one-hour period. During this time the blood sampling for the same chemical determinations was repeated four times at fifteen-minute intervals. Blood sampling for oxygen content and oxygen saturation was interspersed at appropriate intervals during both the control and octanoate periods.

The estimated hepatic blood flow (EHBF) was calculated, for both the control and the octanoate periods, from the corrected infusion rates of bromsulphalein and the arterial-hepatic venous differences of bromsulphalein utilizing the formulae of Bradley, Ingelfinger, Bradley, and Curry (9). Arterial blood concentrations of bromsulphalein did not vary by more than 0.035 mg. per 100 ml. per minute during the control period, nor by more than 0.042 mg. per 100 ml. per minute during any octanoate infusion. It is felt that the formula (9) for correcting the infusion rate of bromsulphalein for such variations applies with reasonable accuracy to the experiments herein reported. Bromsulphalein clearance is an expression of the ratio of the corrected infusion rate to the mean arterial concentration for a given period of study. Bromsulphalein clearance is expressed as per cent of normal, using for reference the data previously accumulated in a series of 63 control subjects (10). The net splanchnic glucose production (NSGP) and splanchnic oxygen consumption (SPO_2) are the products of the hepatic venous-arterial glucose difference in the first case, and the arterial-hepatic venous oxygen difference in the second case, and the respective hepatic blood flow (10, 11).

The total ketones of the blood, expressed as acetone (micromoles per 100 ml. whole blood), were determined by a modification of the Greenberg and Lester method (12) as described below. The net splanchnic ketone production (NSKP) was calculated by multiplying the hepatic venous-femoral arterial difference in ketone concentration by the EHBF. The term "net" has been used to take into account the fact that some of the organs supplied by the splanchnic circulation may be utilizing ketone bodies. The actual production is attributed to the liver.

The per cent of octanoic acid converted to ketones (per cent conversion) was calculated for each patient as follows:

$$\text{Per Cent Conversion} = \frac{\mu\text{M of octanoic acid theoretically required for the mean increase in NSKP per min. per sq. M during the sodium octanoate infusion}}{\mu\text{M octanoic acid infused per min. per sq. M}} \times 100.$$

It should be noted that this expression, per cent conversion, is calculated from the amount of octanoate delivered to the body as a whole, the portion actually perfusing the liver not being known. It cannot be considered as a true estimate of the liver's capacity to form ketone bodies from octanoate.

The following is an outline of the procedure for the determination of blood ketone bodies embodying the principal changes developed in this laboratory during the past five years as well as one suggested by Michaels, Margen, Liebert, and Kinsell (13).[6]

Protein-free blood filtrates are prepared essentially as described by Weichselbaum and Somogyi (14), using 2 ml. of unclotted whole blood (sequestren or heparin) laked in 10 ml. of distilled water, to which are added 4 ml. of 0.15N Ba $(OH)_2$ and 4 ml. of 2.5 per cent $ZnSO_4 \cdot 7H_2O$. The ketone determination is performed on 1 or 2 ml. aliquots of the supernate after centrifugation, depending on the anticipated ketone levels. The entire procedure, except for the photometry, is carried out in 22×150 mm. glass-stoppered pyrex tubes with specially constructed metal clips to hold the stoppers tightly in place during heating. To 2 ml. of supernate (or 1 ml. of supernate plus 1 ml. of distilled water) 0.5 ml. of 13.6N H_2SO_4 is added, the tube shaken and the stopper twisted tightly into place and secured with the metal clip. The tubes are then heated in a mineral oil bath at 110 to 120° C. on an electric hot plate for ten minutes, following which they are cooled to room temperature. In this step the acetoacetate is decarboxylated to acetone. After cooling, 0.5 ml. of 0.4 per cent potassium dichromate is added to each tube and the heating procedure repeated at the same temperature for 30 minutes, resulting in oxidation and decarboxylation of beta-hydroxybutyric acid to acetone. Greenberg and Lester

[6] Modifications made by Mildred G. Engel.

TABLE I

The metabolic effects of intravenously administered sodium octanoate in 16 male subjects *

Pt. Age yrs.	Diagnosis Body surface area	Anxiety during test Remarks	Total ketones (μM acetone/100 ml. blood) Control Fem. art.	Hep. vein	H.V.–F.A.	During sodium octanoate Fem. art.	Hep. vein	H.V.–F.A.	Δ Fem. art.	Δ Hep. vein	Δ H.V.–F.A.	Estimated hepatic blood flow ml. blood/min./M² Control	During sod. oct.	Δ
J. R. 40	Functional G.I. 1.87	2+	16.5	20.3	3.8	47.2	71.5	24.3	+30.6	+51.2	+20.6	673	579	− 94
J. V. 32	Chronic anxiety 2.02	1+	17.0	22.8	5.8	46.5	65.8	19.3	+29.5	+43.0	+13.5	750	713	− 37
T. Mc. 25	? Mild rheumatoid arthritis 1.72	1+	4.8	7.6	2.8	39.2	67.5	28.3	+34.4	+59.9	+25.5	576	515	− 61
A. P. 34	Chr. duod. ulcer 1.84	1+	16.0	19.0	3.0	38.0	61.5	23.5	+22.0	+42.5	+20.5	678	774	+ 96
R. M. 27	Post U.R.I. 1.74	1+	9.2	10.7	1.5	38.2	62.0	23.8	+29.0	+51.3	+22.3	708	660	− 48
E. S. 30	Duodenal ulcer 1.55	1+	10.5	14.8	4.3	65.2	87.3	22.1	+54.7	+72.5	+17.8	974	797	−177
W. J. 23	Carcinoma sphenoid sinus 1.84	1+	14.8	22.0	7.2	43.2	69.5	26.3	+28.4	+47.4	+19.0	923	782	−141
E. S. 62	Chronic sinusitis 1.77	1+	9.0	12.0	3.0	32.0	61.0	29.0	+23.0	+49.0	+26.0	785	708	− 77
J. O'N. 58	Mixed psycho-neurosis 1.72	1+	19.5	25.5	6.0	50.2	67.5	17.3	+30.7	+42.0	+11.3	713	686	− 27
G. W. 36	Headache 1.56	1+	10.5	12.3	1.8	47.0	69.5	22.5	+36.5	+57.2	+20.7	906	784	−122
J. T. 25	Headache 1.65	2+ 6 hr. fast	14.5	23.0	8.5	44.5	73.0	28.5	+30.0	+50.0	+20.0	510	594	+ 84
T. J. 33	? Cord tumor 1.67	1+	6.5	9.8	3.3	26.0	42.5	16.5	+19.5	+37.7	+18.2	1,027	1,227	+200
O. A. 28	Post erythema nodosum 1.85	1+	14.5	17.8	3.3	32.8	50.0	17.2	+18.3	+32.2	+13.9	986	827	−159
J. V. 43	Duod. ulcer 1.81	1+	7.0	6.5	−0.5	31.3	47.8	16.5	+24.3	+41.3	+17.0	698	608	− 90
D. B. 42	Brachial plexus palsy 1.64	1+ 6 hr. fast	31.5	39.0	7.5	59.3	83.0	23.7	+27.8	+44.0	+16.2	890	668	−222
J. W. 48	Functional G.I. 1.59	2+	24.0	32.0	8.0	40.5	58.3	17.8	+16.5	+26.3	+ 9.8	840	788	− 52
Mean Age 37 Range 23–62	Mean S.A. 1.74	Mean S.E. S.D. P.	14.1 1.73 6.92	18.4 2.24 8.96	4.3 0.62 2.46 <0.01	42.6 2.56 10.25	64.8 2.94 11.79	22.2 1.10 4.40 <0.01	+28.5 2.26 9.01 <0.01	+46.4 2.83 11.31 <0.01	+17.9 1.19 4.77 <0.01	790 38.0 151.7	732 40.1 160.4	− 58.0 27.15 108.6 <0.1 >0.05

* The means shown for each subject regarding Total Ketone Levels, Hepatic Vein–Femoral Artery differences, EHBF, NSKP, Femoral Artery Glucose and NSGP represent the average of four interval samples and four separate calculations. The means for the SpO_2 represent the average of two interval samples and two separate calculations.

carried out the above reactions as a single step with acid dichromate, but Michaels, Margen, Liebert, and Kinsell found that there were unpredictable losses of acetoacetate and poor recovery of beta-hydroxybutyrate on heating in acid-dichromate and hence they introduced the two-step procedure. Our experience confirmed that of the latter investigators so this modification was incorporated.

Appropriate acetone standards containing 2 to 5 micrograms per ml., and prepared according to the method outlined by Hawk, Oser, and Summerson (15), are then placed in glass stoppered tubes to which have been added 0.5 ml. of 13.6 N H_2SO_4 and 0.5 ml. of 0.4 per cent potassium dichromate. Blanks are prepared with distilled water. One half ml. of 5 per cent anhydrous sodium sulfite, prepared fresh daily, is added to all tubes

TABLE I—*Continued*

The metabolic effects of intravenously administered sodium octanoate in 16 male subjects

Net splanchnic ketone production *μM acetone/min./M²*			Net splanchnic oxygen consumption *ml./min./M²*			% conversion sod. oct. to ketones	Femoral artery glucose *mg./100 ml. blood*			Net splanchnic glucose production *mg./min./M²*			% BSP clearance		
Control	During sod. oct.	Δ	Control	During sod. oct.	Δ		Control	During sod. oct.	Δ	Control	During sod. oct.	Δ	Control	During sod. oct.	Δ
26	140	+114	33	37	+ 4	15	80	78	−2	50	50	0	76	72	−4
43	137	+ 94	37	36	− 1	13	92	90	−2	61	44	−17	78	77	−1
16	145	+129	28	34	+ 6	15	78	77	−1	49	44	− 5	82	82	0
22	180	+158	28	39	+11	20	83	80	−3	64	60	− 4	75	70	−5
5	158	+153	35	35	0	18	76	74	−2	61	46	−15	76	75	−1
42	170	+128	31	36	+ 5	14	83	83	0	83	44	−39	73	68	−5
66	203	+137	46	40	− 6	17	81	78	−3	96	72	−24	86	83	−3
24	204	+180	—	—	—	22	105	100	−5	108	87	−21	64	—	—
43	119	+ 76	38	42	+ 4	9	79	78	−1	48	58	+10	83	75	−8
15	176	+161	32	36	+ 4	17	82	76	−6	52	66	+14	109	109	0
40	173	+133	34	42	+ 8	16	87	84	−3	71	58	−13	109	103	−6
34	163	+129	30	45	+15	15	75	71	−4	68	98	+30	97	95	−2
32	142	+110	56	58	+ 2	14	90	83	−7	90	121	+31	98	98	0
0 ?	101	+101	46	42	− 4	13	84	80	−4	68	41	−27	86	81	−5
67	158	+ 91	39	42	+ 3	10	90	86	−4	67	52	−15	81	77	−4
68	159	+ 91	29	40	+11	10	88	88	0	52	99	+47	77	68	−9
34.0	158.0	+124	36.1	40.3	+ 4.2	14.9	84.6	81.6	−3.0	68.0	65.0	− 3.0	84.4	82.2	−3.4
5.20	6.86	7.36	2.05	1.52	1.46	0.88	1.87	1.76	0.5	4.47	6.0	5.48	3.19	3.32	0.73
20.86	27.46	41.4	7.94	5.83	5.66	3.46	7.46	7.05	2.01	17.92	24.08	21.82	12.81	13.04	2.85
		<0.01			<0.02				<0.01			>0.5			<0.01
					>0.01										

to destroy the excess potassium dichromate. After mixing add 2 ml. of 2,4 dinitrophenylhydrazine (0.1 per cent in 2N HCl), shake and then introduce exactly 2.0 ml. of reagent grade carbon tetrachloride [7] into each tube. A calibrated automatic filling, reservoir type of pipette is the most convenient for this purpose.

[7] Each new lot of carbon tetrachloride should be tested since some yield high blanks with 2,4 dinitrophenylhydrazine. These should not be used.

The tubes are then placed in a large mechanical shaker,[8] and are shaken for 10 minutes. The shaking can be carried out using a standard general purpose reciprocating type shaker [9] at top speed, but one hour is necessary for complete extraction. After shaking, the super-

[8] International Equipment Company, Bottle Shaker, Size 2, with specially designed screw-topped adapters fitted to hold 40 of the glass-stoppered tubes.

[9] A. H. Thomas Company, No. 8917A.

natant acid solution is drawn off with suction and discarded. The carbon tetrachloride layer is washed with distilled water and the water layer sucked off. This washing is repeated a second time after which 3 ml. of 0.5N NaOH are added to each sample. The tubes are shaken again for 3 minutes in the standard general purpose shaker and the contents then are poured into small standardized tubes (Wassermann tubes) and the color in the lower carbon tetrachloride layer read at 420 millimicrons in a Coleman Jr. Spectrophotometer.

Seasonal temperature changes may cause changes in the blanks and standards because of the nature of the solvent, but in an air-conditioned laboratory this has been no problem. It is important to avoid the use of acetone in the same laboratory where these determinations are being carried out, as acetone is readily taken up from the air by the filtrates and certain of the reagents. It should also be noted that acetone can also be picked up from other laboratories by air conditioning or other ventilating devices. In our experience, this has been a major cause for gross variations in the results.

The reproducibility of the ketone method, tested by comparing 23 random duplicate values, was found to be within 6 per cent for the control range of levels and within 9 per cent for the elevated levels induced by sodium octanoate infusion.

The modification, according to Michaels, Margen, Liebert, and Kinsell (13) noted above, was introduced after the present study had already been initiated, but seemed worthy of adoption since it reputedly gave higher yields of acetone from beta-hydroxybutyrate. On comparing the single acid-dichromate procedure with the two-step heating method (13) in identical samples of filtrates from nine subjects in this study, the latter modification resulted in a small but significantly ($P < 0.01$) greater yield of total blood ketones. The difference increased as the total concentration of ketones rose. However, when ketone levels of different groups of subjects were compared in relation to the two techniques the means were practically identical. Thus, the biological scatter apparently exceeded the small difference in the two methods. For the purpose of this study it seemed sufficiently accurate to incorporate the data from the initial series of subjects whose blood ketones had been determined without the Michaels modification with those from the more recent group of subjects where the change had been made.

One final question arose in regard to the ketone method, namely whether the higher levels obtained during the sodium octanoate infusion could possibly be due to the formation and release by the liver of beta-keto-octanoic acid. Beta-keto-octanoic acid should form a colored hydrazone with 2,4 dinitrophenylhydrazine and hence interfere with the determination of ketone bodies. If beta-keto-octanoic acid were present in filtrates from blood collected during the sodium octanoate period, it should give the color reaction with acid 2,4 dinitrophenylhydrazine after gentle heating to evaporate all the free acetone, and without treating the filtrates with acid and dichromate. Accordingly, filtrates obtained from each

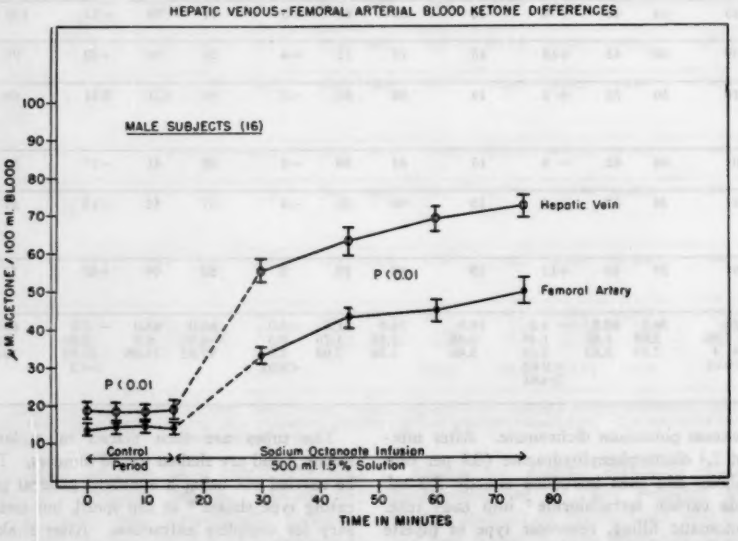


FIG. 1. HEPATIC VENOUS AND FEMORAL ARTERIAL BLOOD KETONE LEVELS IN 16 MALE SUBJECTS (MEAN ± S.E.)

The p values refer to the mean hepatic vein—femoral artery ketone differences during the entire control and experimental periods.

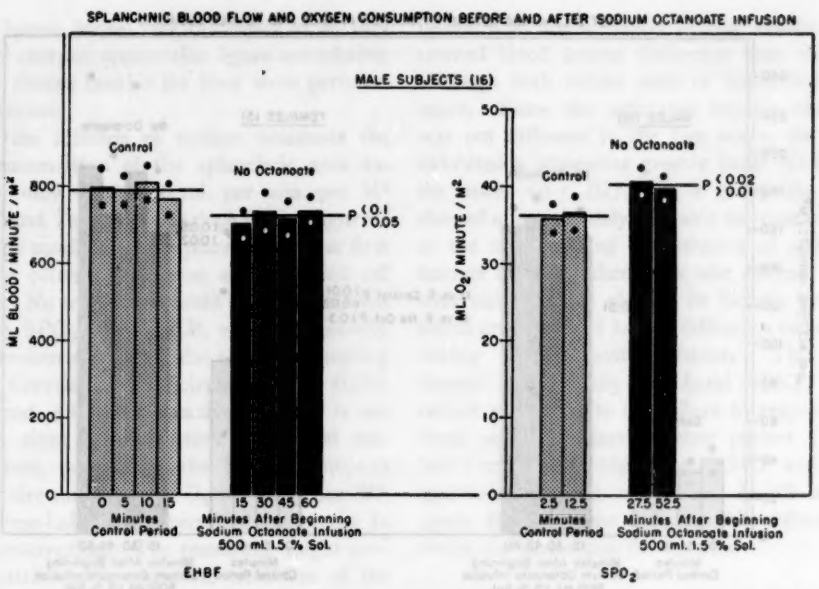


FIG. 2. ESTIMATED HEPATIC BLOOD FLOW AND NET SPLANCHNIC OXYGEN CONSUMPTION IN 16 MALE SUBJECTS (MEAN ± S.E.)

The long horizontal lines represent the mean values for the entire control and experimental periods, and the p values indicate the statistical significance between these means. In the EHBF studies, although there was no significant difference in the mean values for the control and experimental period, the flow during the first 15 minutes after Na octanoate was significantly decreased ($p < 0.01$).

of three subjects after sodium octanoate were pooled and tested. No color developed, eliminating the possibility of beta-keto-octanoic acid being present in any significant quantity.

RESULTS

Male series

The data on the 16 male subjects are recorded in Table I and Figures 1, 2, 3, and 4. The mean control ketone level in femoral arterial blood was 14.1 ± 1.73 μM per cent (standard error of the mean) and in hepatic venous blood 18.4 ± 2.24 μM per cent. The mean difference of 4.3 ± 0.62 μM per cent represents a statistically significant ($p < .01$) elevation of the hepatic venous level over the femoral arterial value. In three subjects (R. M., G. W., and J. V.) the hepatic venous ketone level was not significantly different from the corresponding femoral arterial level, as judged by the limit of error of the ketone method. In all other subjects there was a distinctly measurable hepatic venous femoral arterial blood ketone difference. This is illustrated in Figure 1 which depicts the mean values at each period of sampling.

Following the infusion of 500 ml. of a 1.5 per cent solution of sodium octanoate there was a brisk increase in the ketone levels of hepatic venous blood (64.8 ± 2.94 μM per cent), in femoral arterial blood (42.6 ± 2.56 μM per cent), and in the hepatic venous-femoral arterial difference (22.2 ± 1.10 μM per cent). The increase over the control in each case was highly significant statistically ($p < .01$). The increment in the hepatic venous ketone value was much greater than that of femoral arterial blood. This situation must represent a greatly augmented output of ketone bodies by the splanchnic area, provided there was no great fall in the estimated hepatic blood flow. The data recorded in Table I and Figure 2 indicate that, although there was a quite small but significant fall ($p < .01$) in the esti-

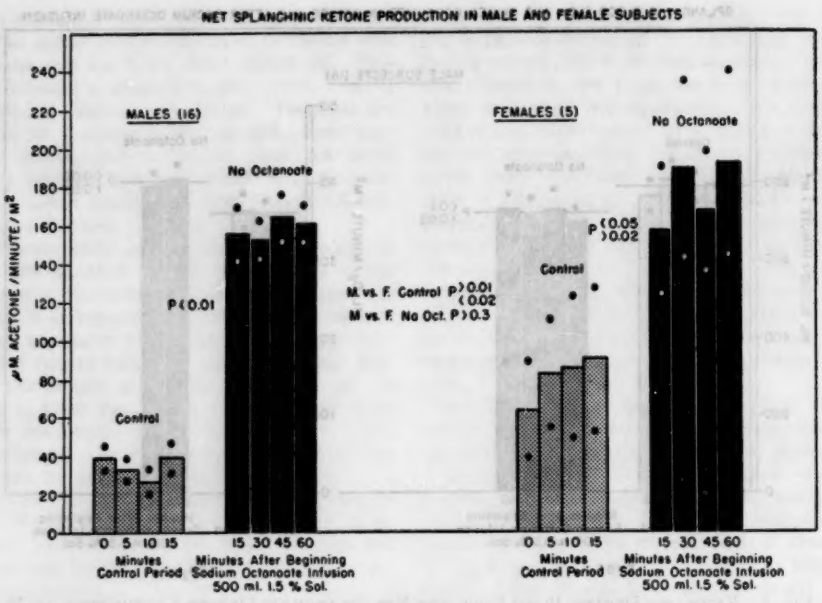


FIG. 3. NET SPLANCHNIC KETONE PRODUCTION IN 16 MALE AND 5 FEMALE SUBJECTS (MEAN ± S.E.)

The p values relate to the mean NSKP during the entire control and experimental periods.

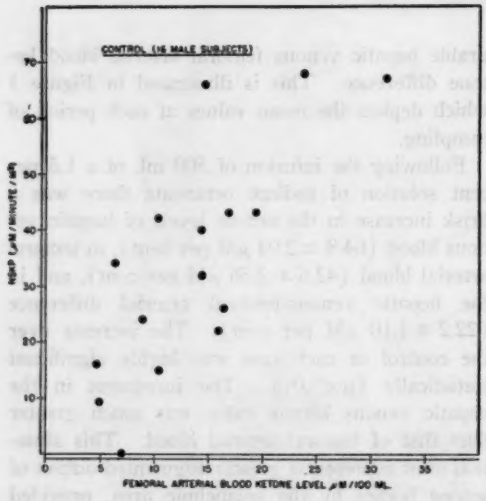


FIG. 4. RELATIONSHIP OF FEMORAL ARTERIAL BLOOD KETONE LEVELS TO NSKP IN 16 MALE SUBJECTS DURING THE CONTROL PERIOD

Correlation coefficient, r = + 0.71.

mated hepatic blood flow during the first 15 minutes of sodium octanoate infusion, this was not progressive.

From the estimated hepatic blood flow and the hepatic venous-femoral arterial blood ketone difference it is possible to calculate the net splanchnic ketone production (NSKP). This appears in Table I and Figure 3. During the control period there was a mean NSKP amounting to 34.0 ± 5.20 μM acetone per min. per M^2 and this increased to 158 ± 6.86 μM acetone per min. per M^2 during the infusion of sodium octanoate. The mean increase of 124 ± 7.36 μM acetone per min. per M^2 is, of course, highly significant statistically.

A close correlation ($R = +0.71$, $p < .01$) was found between the arterial blood ketone levels and the NSKP during the basal period (Figure 4). No such correlation could be demonstrated between the arterial blood ketone values and the NSKP during the period of sodium octanoate infusion.

The per cent of infused sodium octanoate con-

verted to ketone bodies was calculated to be 14.9 ± 0.88 per cent, an appreciable figure considering that many tissues besides the liver were perfused by the octanoate.

During the infusion of sodium octanoate the oxygen consumption of the splanchnic area increased by only 4.2 ± 1.46 ml. per min. per M^2 (Figure 2 and Table I). As depicted in Figure 2 most of this small rise took place during the first half of the octanoate infusion and then fell off somewhat. No correlation could be made between the rise in SPO_2 and NSKP, either collectively or when broken down into the separate sampling periods. Correlation coefficients were + 0.254, + 0.207, and + 0.353, respectively. This is not surprising, since the production of 124 μM acetone per min. per M^2 by the 16 male subjects would require only 1.9 ml. O_2 per min. per M^2, or about one-half of the recorded increment in oxygen consumption. The remainder might possibly be attributed to complete oxidation of the fatty acid, which would require more oxygen.

Measurement of the femoral arterial blood glucose levels demonstrated a small fall (3.0 ± 0.5 mg. per cent) during the fatty acid infusion (Table I). This was statistically significant ($p < .01$) and presumably can be attributed to hemodilution. The net splanchnic glucose production was not modified by the infusion.

Bromsulphalein clearances were generally within the normal range or mildly subnormal, as could be expected in slightly ill subjects, during the control period. They exhibited a small, but significant ($p < .01$) decrement during the infusion of the sodium octanoate (Table I). The mechanism of this fall is not immediately apparent unless the simultaneous oxidation of fatty acids by the liver interferes with the mechanism for removing bromsulphalein.

Female subjects

(Table II, Figures 3 and 5.) Five female subjects were studied and compared to the males, bringing out certain minor differences between the two groups. However, in view of the small sample these differences must be accepted with caution, particularly since they may be accounted for largely by two subjects. As a group the females showed a somewhat higher hepatic venous blood ketone level and a greater hepatic venous-femoral arterial blood ketone difference than the males, although both values were of borderline significance. Since the estimated hepatic blood flow was not different in the two sexes, the females exhibited a somewhat greater basal NSKP than the males ($p < .02$). As a group the females showed approximately the same increase in NSKP as the males during the infusion of sodium octanoate although there was one subject (F. S.) who exhibited no change in hepatic venous-femoral arterial blood ketone difference or in NSKP during the octanoate infusion. This patient showed an unusually high basal NSKP, but one cannot relate this to the failure to respond to sodium octanoate since another patient (A. M.) had a comparably high basal NSKP and still responded briskly to octanoate. In all other respects there was no demonstrable difference between the males and the females.

DISCUSSION

The primary objective of this study was to develop a method which would quantitate splanchnic ketone production in man. It was found that after an overnight fast a small but significant endogenous production of ketone bodies was demonstrable in the splanchnic area and that this was notably increased by an intravenous infusion of a known ketone precursor, sodium octanoate. The sudden influx of a comparatively large amount of fatty acid increased hepatic ketogenesis presumably because it could not be converted to fat rapidly enough and hence was degraded to acetoacetyl-CoA and/or acetyl-CoA. The latter was not completely oxidized *via* the Krebs cycle and hence spilled over into the blood stream as acetoacetate.

In this study the ketosis induced by sodium octanoate occurred promptly. This indicates that the numerous enzymatic steps, which are required for fatty acid catabolism and the hepatic release of free acetoacetate, are accomplished rapidly and without delay in the intact human (2–5, 16–21). The findings of Lipsky, Alper, Rubini, Van Eck, and Gordon (22) are in disagreement in that these investigators found a delay of two to four hours in the ketosis induced by the administration of intravenous sodium acetate to humans. How-

TABLE II

The metabolic effects of intravenously administered sodium octanoate in five female subjects

Pt.	Diagnosis	Body surface area	Anxiety during test	Remarks	Total ketones (μM acetone/100 ml. blood) Control: Fem. art.	Control: Hep. vein	Control: H.V.−F.A.	During sodium octanoate: Fem. art.	During sodium octanoate: Hep. vein	During sodium octanoate: H.V.−F.A.	Δ: Fem. art.	Δ: Hep. vein	Δ: H.V.−F.A.	Estimated hepatic blood flow ml. blood/min./M² Control	During sod. oct.	Δ
yrs.																
C. H. 38	Labyrinthitis	1.70	1+		17.8	24.5	6.7	48.0	67.8	19.8	+30.2	+43.3	+13.1	697	588	−109
F. S. 30	Mixed psychoneurosis	1.71	2+		13.8	34.3	20.5	30.8	48.6	17.8	+17.0	+14.2	− 2.8	892	934	+ 42
A. M. 31	Chronic otitis media	1.72	2+		28.5	46.5	18.0	76.5	121.0	44.5	+48.0	+74.7	+26.7	828	602	−226
W. R. 31	Headache	1.71	1+		18.0	20.2	2.2	50.5	67.5	17.0	+32.5	+47.3	+14.8	1,130	1,150	+ 20
C. R. 47	Mild rheumatoid arthritis	1.69	2+		18.0	22.0	4.0	50.0	72.0	22.0	+32.0	+50.0	+18.0	687	574	−113
Mean age 35.4	Mean S.A.	1.71	Mean		19.2	29.5	10.3	51.2	75.4	24.2	+31.9	+45.9	+14.0	847	770	− 77.0
			S.E.		2.45	4.89	3.58	7.29	12.09	5.17	4.91	9.60	4.53	80.7	116.5	49.0
			S.D.		5.49	10.95	8.02	16.34	27.09	11.59	11.0	21.50	10.2	180.7	261.0	109.8
Range 30–47			P				<0.05			<0.01			<0.1			
							>0.02				<0.01	<0.01	>0.05			>0.1
Statistical* comparison with male series			t =		1.53	2.2	2.6	1.5	1.3	0.6	0.7	0.5	1.1	1.19	0.57	0.35
			p =		>0.1	>0.02 <0.05	>0.01 <0.02	>0.1	>0.2	>0.5	>0.4	>0.5	>0.2	>0.2	>0.5	>0.5

* The mean figures represent averages as described for male series in Table I. The statistical comparison between the male and female series is presented in the lower section of the table.

ever, Ciaranfi and Fonnesu (23), in a recent report, demonstrated an immediate small rise in peripheral blood ketones after infusing sodium acetate intravenously into normal and depancreatized dogs.

Other published observations on the measurement of ketone production in humans are those of Bondy, Bloom, Whitner, and Farrar (24) who reported on splanchnic ketone formation during diabetic acidosis in humans, and of Lipsky and his associates (22) who, as mentioned above, reported on ketone production induced by the infusion of sodium acetate into diabetic patients. Although the techniques applied are similar, comparison of their results with the present study is of limited value since their data were few in number and all were on diabetic subjects. Crandall, Ivy, and Ehni (25) in 1941 estimated hepatic ketogenesis from the hepatic venous-arterial blood ketone levels in fasting and fat-fed angiostomized dogs without directly measuring hepatic blood flow. They were unable to detect any hepatic formation of ketone bodies in dogs fasted less than three days. They attributed their failure to the inaccuracy of their method of ketone analysis.

There are two factors which must be taken into consideration in the interpretation of the results of the present study. Hinkle, Conger, and Wolf (26) have stressed anxiety as a potentiator of ketosis in diabetic patients. It is difficult to state conclusively whether anxiety may have played a role in determining the control NSKP in the present study. All routine steps were taken to minimize anxiety, but certainly it was not eliminated. Nevertheless, the constancy of the results in the large male series makes it seem unlikely that a factor as variable as anxiety could be playing a significant role in the results.

The second factor to be considered is alkalosis which has been found in animals (27) to increase fasting ketonemia. Moreover, Lipsky and his colleagues (22) have reported increased hepatic ketogenesis after infusing sodium bicarbonate or sodium acetate into humans. It is very doubtful, however, that the 500 ml. of sodium octanoate (55 mEq. of sodium) used in the present study would produce an alkalosis comparable to that observed by other workers (22) after the administration of 238 mEq. of sodium as bicarbonate and acetate.

The arterial blood ketone level represents a balance between splanchnic production and peripheral utilization. When the arterial blood level of ketones is constant, it can be assumed that splanch-

TABLE II—*Continued*

The metabolic effects of intravenously administered sodium octanoate in five female subjects

Net splanchnic ketone production μM *acetone/min./M²*			Net splanchnic oxygen consumption *ml./min./M²*			% conversion sod. oct. to ketones	Femoral artery glucose *mg./100 ml. blood*			Net splanchnic glucose production *mg./min./M²*			% BSP clearance		
Control	During sod. oct.	Δ	Control	During sod. oct.	Δ		Control	During sod. oct.	Δ	Control	During sod. oct.	Δ	Control	During sod. oct.	Δ
47	116	+ 69	29	34	+ 5	8	83	81	−2	56	67	+11	82	82	0
175	166	− 9	41	38	− 3	0	98	93	−5	89	80	− 9	85	87	+2
149	271	+122	33	23	−10	14	72	69	−3	93	68	−25	62	63	+1
25	204	+179	42	55	+13	21	77	76	−1	59	63	+ 4	96	96	0
32	123	+ 91	32	38	+ 6	11	80	77	−3	54	44	−10	62	57	−5
86.0	176.0	+ 90.0	35.2	37.6	+ 2.4	10.8	82	79.2	−2.8	70.2	64.4	− 5.8	77.4	77.0	−0.4
31.5	28.5	30.9	2.42	5.13	3.72	3.45	4.39	3.95	0.66	8.54	5.83	6.21	6.74	7.33	2.7
71.6	63.8	69.2	5.38	11.50	8.31	7.73	9.84	8.85	1.48	19.14	13.06	13.92	15.0	16.43	1.2
		<0.05							<0.02						
		>0.02			>0.5				>0.01			>0.4			>0.5
2.68	0.92	0.95		0.7		1.68	0.62	1.1		0.29		0.28	1.05	0.728	2.04
>0.01	>0.3	>0.3	>0.5	>0.4	>0.5	>0.1	>0.5	>0.2	>0.5	>0.5	>0.5	>0.5	>0.3	>0.4	>0.05
<0.02															<0.1

nic production and peripheral utilization are equal. Such a state of affairs existed in this study in the control period, and hence the net splanchnic ketone production, which was estimated directly from splanchnic blood flow and splanchnic blood ketone difference, can be taken as a measure of fasting ketone utilization during this period. It will be recalled that during the control period NSKP varied directly with the arterial blood ketone concentration. Thus utilization was higher when arterial levels were higher. Gammeltoft (28) has made a similar observation. The rising

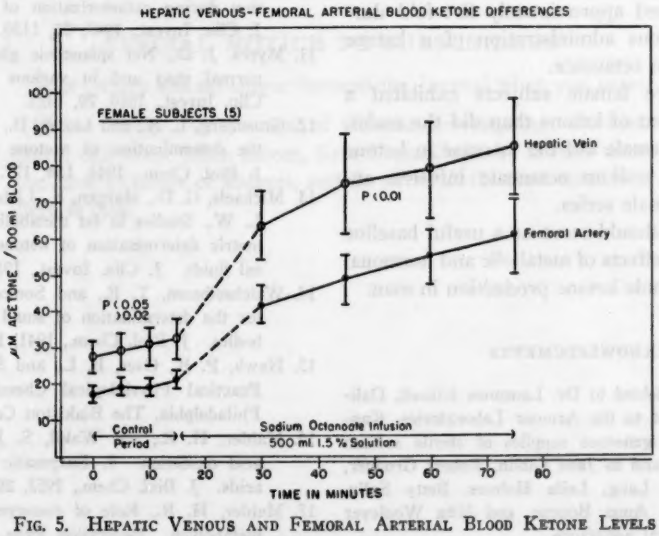


FIG. 5. HEPATIC VENOUS AND FEMORAL ARTERIAL BLOOD KETONE LEVELS (MEAN ± S.E.)

and falling arterial blood ketone levels after octanoate infusion prevent one from translating ketone production to ketone utilization during that period.

Too great emphasis should not be placed on the differences in ketogenesis between the male and female groups. The series of five females is small and the irregularities were due chiefly to two of the five. However, other investigators have noted the factor of sex in ketosis, and hence further study is indicated. Peters (29) noted that female humans are more susceptible to ketosis, and Deuel and Hallman (30) observed a greater ketonemia following a high fat diet in fasted female rats than in pair-fed males. Friedemann (31) on the other hand noted that male monkeys fed a high fat diet excreted more ketones than females.

SUMMARY

1. Utilizing the technique of hepatic vein catheterization, a method has been developed for measuring directly the splanchnic production of ketones in man and permitting an indirect assessment of peripheral ketone utilization.

2. Employing this method a small but significant endogenous splanchnic ketone production was measured in fourteen out of sixteen mildly ill male subjects in the post-absorptive state. This was increased approximately five-fold during the intravenous administration of a ketone precursor, sodium octanoate.

3. Two of five female subjects exhibited a higher basal output of ketone than did the males, but in only one female did the increase in ketone production after sodium octanoate infusion exceed that of the male series.

4. These data should serve as a useful baseline in evaluating the effects of metabolic and hormonal factors on splanchnic ketone production in man.

ACKNOWLEDGMENTS

We are much indebted to Dr. Laurance Kinsell, Oakland, California, and to the Armour Laboratories, Kankakee, Illinois, for generous supplies of sterile sodium octanoate solution, and to Jane Giblin, Robert Grodner, Irving Kalb, Rita Lang, Leila Holmes, Betty Sally, Dorothy Frederick, Anna Bourne, and Nita Woolever for valuable technical assistance.

REFERENCES

1. Lipmann, F., Biosynthetic mechanisms. Harvey Lectures, 1948–1949, **44**, 99.
2. Lynen, F., and Ochoa, S., Enzymes of fatty acid metabolism. Biochem. et Biophys. Acta, 1953, **12**, 299.
3. Lynen, F., Acetyl coenzyme A and the "fatty acid cycle." Harvey Lectures, 1952–1953, **48**, 210.
4. Green, D. E., Oxidation and synthesis of fatty acids in soluble enzyme systems of animal tissues. Clin. Chem., 1955, **1**, 53.
5. Lipmann, F., Consideration of the role of coenzyme A in some phases of fat metabolism *in* Fat Metabolism, A Symposium, V. A. Najjar, Ed., Baltimore, Johns Hopkins Press, 1954.
6. Engel, F. L., The endocrine control of metabolism. Bull. New York Acad. Med., 1953, **29**, 175.
7. Weinhouse, S., Factors involved in the formation and utilization of ketone bodies. Major Metabolic Fuels. Brookhaven Symposia in Biology, 1952, **5**, 201.
8. Margen, S., Michaels, G. D., Boling, L. A., and Kinsell, L. W., Hormonal regulation of fat metabolism. II. Effects of ACTH and certain steroid hormones upon the utilization of infused acetoacetate and octonoic acid. Proc. 2nd Clinical ACTH Conf., J. R. Mote, Ed., Philadelphia, Blakiston Co., 1951, vol. 1, p. 318.
9. Bradley, S. E., Ingelfinger, F. J., Bradley, G. P., and Curry, J. J., The estimation of hepatic blood flow in man. J. Clin. Invest., 1945, **24**, 890.
10. Myers, J. D., The hepatic blood flow and splanchnic oxygen consumption of man—their estimation from urea production and bromsulphalein excretion during catheterization of the hepatic veins. J. Clin. Invest., 1947, **26**, 1130.
11. Myers, J. D., Net splanchnic glucose production in normal man and in various disease states. J. Clin. Invest., 1950, **29**, 1421.
12. Greenberg, L. A., and Lester, D., A micromethod for the determination of acetone and ketone bodies. J. Biol. Chem., 1944, **154**, 177.
13. Michaels, G. D., Margen, S., Liebert, G., and Kinsell, L. W., Studies in fat metabolism. I. The colorimetric determination of ketone bodies in biological fluids. J. Clin. Invest., 1951, **30**, 1483.
14. Weichselbaum, T. E., and Somogyi, M., A method for the determination of small amounts of ketone bodies. J. Biol. Chem., 1941, **140**, 5.
15. Hawk, P. B., Oser, B. L., and Summerson, W. H., Practical Physiological Chemistry, 12th edition, Philadelphia, The Blakiston Co., 1947, p. 875.
16. Mahler, H. R., and Wakil, S. J., Studies on fatty acid oxidation. I. Enzymatic activation of fatty acids. J. Biol. Chem., 1953, **204**, 453.
17. Mahler, H. R., Role of coenzyme A in fatty acid metabolism. Federation Proc., 1953, **12**, 694.

18. Lehninger, A. L., and Greville, G. D., The enzymic oxidation of d- and l-β-hydroxybutyrate. Biochem. et Biophys. Acta, 1953, **12**, 188.
19. Beinert, H., Studies on the fatty acid oxidizing system of animal tissues. II. β-ketoacyl derivatives of coenzyme A. J. Biol. Chem., 1953, **205**, 575.
20. Stern, J. R., Coon, M. J., and del Campillo, A., Acetoacetyl coenzyme A as intermediate in the enzymatic breakdown and synthesis of acetoacetate. J. Am. Chem. Soc., 1953, **75**, 1517.
21. Green, D. E., Mii, S., and Mahler, H. R., Studies on the fatty acid oxidizing system of animal tissues. III. Butyryl coenzyme A dehydrogenase. J. Biol. Chem., 1954, **206**, 1.
22. Lipsky, S. R., Alper, B. J., Rubini, M. E., Van Eck, W. F., and Gordon, M. E., The effects of alkalosis upon ketone body production and carbohydrate metabolism in man. J. Clin. Invest., 1954, **33**, 1269.
23. Ciaranfi, E., and Fonnesu, A., Time-course of injected acetate in normal and depancreatized dogs. Biochem. J., 1954, **57**, 171.
24. Bondy, P. K., Bloom, W. L., Whitner, V. S., and Farrar, B. W., Studies of the role of the liver in human carbohydrate metabolism by the venous catheter technic. II. Patients with diabetic ketosis, before and after the administration of insulin. J. Clin. Invest., 1949, **28**, 1126.
25. Crandall, L. A., Jr., Ivy, H. B., and Ehni, G. J., Hepatic acetone body production in the dog during fasting and fat feeding. Am. J. Physiol., 1940, **131**, 10.
26. Hinkle, L. E., Jr., Conger, G. B., and Wolf, S., Studies on diabetes mellitus: the relationship of stressful life situations to the concentration of ketone bodies in the blood of diabetic and non-diabetic humans. J. Clin. Invest., 1950, **29**, 754.
27. MacKay, E. M., Wick, A. N., Carne, H. O., and Barnum, C. P., The influence of alkalosis and acidosis upon fasting ketosis. J. Biol. Chem., 1941, **138**, 63.
28. Gammeltoft, A., The significance of ketone bodies in fat metabolism. I. Concentration of ketone bodies in the arterial and venous blood in human subjects during starvation. Acta physiol. Scandinav., 1949, **19**, 270.
29. Peters, J. P., Diabetic acidosis. Metabolism, 1952, **1**, 223.
30. Deuel, H. J., Jr., and Hallman, L. F., Studies on ketosis. XIX: Further studies on endogenous ketonemia in the rat. J. Biol. Chem., 1941, **140**, 545.
31. Friedemann, T. E., Ketosis in primates. II. The ratio of β-hydroxybutyric acid to acetoacetic acid in the blood and urine. J. Biol. Chem., 1942, **142**, 635.

SPECIAL NOTICE TO SUBSCRIBERS

Post Offices will no longer forward the Journal when you move.

Please notify The Journal of Clinical Investigation, Business Office, 622 West 168th Street, New York 32, N. Y. at once when you have a change of address, and do not omit the zone number if there is one.

ABNORMALITIES OF CARBOHYDRATE METABOLISM OF RED CELLS IN HEREDITARY SPHEROCYTOSIS [1, 2]

By THOMAS A. J. PRANKERD,[3] KURT I. ALTMAN, AND LAWRENCE E. YOUNG

(From the Departments of Medicine and Radiation Biology, the University of Rochester School of Medicine and Dentistry, and the Medical Clinics of the Strong Memorial Hospital, Rochester, N. Y.)

(Submitted for publication September 16, 1954; accepted April 6, 1955)

We have recently described a method for investigating glycolysis of red cells by using P^{32}-labeled orthophosphate to measure the rate of turnover of various organic phosphate esters (1). Observations on normal cells incubated under various conditions have been made with this method and described in separate reports (2, 3). Because inborn errors of metabolism have been demonstrated in a variety of hereditary diseases, this method has been applied especially to the study of red cells from patients with certain congenital hemolytic disorders. We have focused our attention particularly upon hereditary spherocytosis (to be abbreviated as HS) and will report results obtained from patients with this disorder in this paper. Our findings in other hemolytic states, both congenital and acquired, will be described later.

METHODS

The methods of estimating the rate of incorporation of radioactive orthophosphate into phosphate esters of the red cell have been described previously in detail (1, 2). Freshly drawn heparinized blood was incubated at 37.5° C. with 0.05 to 0.15 ml. of 0.0007 N $NaH_2P^{32}O_4$ having approximately 10^5 counts per minute (c.p.m.) and at appropriate intervals the phosphorus compounds were extracted with trichloracetic acid from samples of red cell stroma and from the remaining intracellular material. From these extracts the phosphorus-containing compounds were isolated by paper chromatography, using the method of Caldwell (4), and the radioactivity and total concentration of phosphorus in each fraction were determined. In most cases estimates of relative specific activity (RSA) were made on samples taken after incubation for two and four hours, since equilibrium is not usually reached before six hours. In the course of other experiments in this laboratory we have found that the concentration of glucose in both normal and HS blood after four hours' incubation at body temperature varies from approximately 10 mg. to 30 mg. per 100 ml. of blood and is sufficient to support cell metabolism. RSA as defined here refers to c.p.m. per μg. P corrected to a standard addition of 10^5 c.p.m. All values for the rate of P^{32} exchange were corrected to a standard hematocrit of 40 per cent and a standard addition of 10^5 c.p.m. per ml. plasma.

The hematocrit of venous blood was determined by the method of Wintrobe (5) and corrected for trapped intercellular plasma by using the graphs of Chaplin and Mollison (6). Chaplin and Mollison (6) and Furth (7) have found little difference between normal and HS cells with regard to trapped plasma after centrifugation in Wintrobe tubes, provided the blood was freshly drawn and the hematocrit was within the range encountered in this study (7). Methods of measuring osmotic and mechanical fragility of red cells in both freshly drawn and incubated defibrinated blood have been described elsewhere (8). Autohemolysis of red cells in sterile defibrinated blood during incubation at body temperature for 48 hours was measured by the method of Selwyn and Dacie (9) with minor modifications. Direct antiglobulin tests were made by the method of Evans and Duane (10).

CLINICAL MATERIAL

In Table I are listed pertinent clinical and hematologic data obtained in 18 cases of chronic spherocytosis. All of these patients showed the typical hematological findings of hereditary spherocytosis (8). The most important of these were: the presence of spherocytes in smears or wet preparations of peripheral blood, increased osmotic and mechanical fragility of the red cells with greater than normal increments in fragility after incubation of the blood for 24 hours at body temperature, marked spontaneous lysis of the red cells during incubation at body temperature for 48 hours, and inagglutinability of the red cells by antiglobulin serum (negative direct Coombs test).

[1] These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and the University of Rochester (NR 102–174) and by the David Smalline and Karl R. Schnepel Memorial Funds. This paper is based in part on work performed under contract with the U. S. Atomic Energy Commission at the University of Rochester Atomic Energy Project.

[2] This report was presented in abbreviated form at the annual meeting of the American Society for Clinical Investigation, Atlantic City, New Jersey, May 3, 1954.

[3] Traveling Fellow of the British Postgraduate Medical Federation, Present Address: University College Hospital, London W.C. 1.

TABLE I

Clinical and hematologic data obtained in 18 cases of spherocytosis at time of studies on red cell metabolism

Case No.	Sex	Age yrs.	Interval since splenectomy	Hematocrit %	Reticulocytes %	Osmotic fragility % NaCl causing 50% hemolysis: Fresh blood	Osmotic fragility % NaCl causing 50% hemolysis: Incubated blood	Number of relatives with spherocytosis
A 1	M	60	pre-spl.	33.0	8.0	0.47	0.65	3
A 2	M	53	pre-spl.	43.0	—	0.52	0.69	3
A 3	F	76	pre-spl.	31.0	3.5	0.47	0.64	4
A 4	M	29	pre-spl.	45.0	3.0	0.47	0.63	1
A 5	F	47	pre-spl.	32.0	17.0	0.48	0.64	3
A 6	M	15	pre-spl.	33.5	5.4	0.49	0.62	1
			2 wks.	42.0	1.1	0.48	0.62	
A 7	M	3	6 wks.	39.5	0.3	—	—	1
A 8	M	41	4 yrs.	51.0	0.5	0.50	0.64	1
A 9	M	16	10 yrs.	48.5	2.4	0.49	0.66	3
A 10	M	28	6 yrs.	49.0	—	0.48	0.66	4
A 11	M	43	3 yrs.	41.0	—	0.50	0.67	1
A 12	F	10	6 yrs.	40.0	0.7	0.47	—	3
A 13	M	8	2 yrs.	39.0	0.6	0.47	—	3
A 14	F	33	10 yrs.	44.5	1.4	0.49	0.64	3
A 15	F	2	1 yr.	36.0	0.6	0.49	—	1
B 1	M	16	14 yrs.	43.0	—	0.47	0.67	0
B 2	M	14	2 yrs.	43.0	—	0.49	0.66	0
B 3	F	15	4 yrs.	40.0	0.7	0.47	0.62	0
				Normal mean (26 persons examined)		0.41 ±0.015	0.51 ±0.03	
Family groups:				(A 1, A 2, A 9)	(A 3, A 10)	(A 4, A 7)	(A 5, A 12, A 13, A 14)	(A 6, A 8)

Hematologic abnormalities of the red cells were found to persist in the 13 splenectomized patients who were re-examined after intervals of from 2 weeks to 14 years following operation. Histological examination of the spleen in each of the 13 cases revealed findings in accord with a diagnosis of hereditary spherocytosis (11–13).

One or more relatives of each of the first 15 patients listed in Table I (group A) was shown unequivocally to have hematologic abnormalities typical of hereditary spherocytosis. No hematologic abnormality could be demonstrated in either parent or in any of the other available relatives of the last 3 patients listed in Table I (group B). None of these 3 patients has yet produced offspring. These and other atypical cases of chronic spherocytosis will be dealt with in detail in a separate paper. We have included the cases of group B in this report because the hematologic findings before and after splenectomy followed the same pattern as that of the 15 cases of group A.

RESULTS OF STUDIES ON RED CELL METABOLISM

Studies on normal red cells

Under the conditions of these experiments (2) the exchange of P^{32} between plasma and red cells from hematologically normal persons was found to have a rate constant of 0.24 ± 0.1 hr.$^{-1}$. This constant represents the fall in plasma P^{32} in arbitrary units per hour, and is calculated from the standard equation for a first order process: $K = 1/t \ln (C_o/C_t)$. This is actually a composite constant representing the disappearance of P^{32} from the plasma and incorporation into cell esters (2). A constant interrelationship was demonstrated among the RSA of various intracellular phosphate esters (Figure 1), in that 2,3-diphosphoglycerate (2,3-DPG) had a higher RSA than adenosine triphosphate (ATP)[4] and the RSA of these compounds exceeded that of inorganic phosphate (IP) except at the very beginning of incubation and at equilibrium (1, 2). The relation of the RSA of 2,3-DPG and ATP is compatible with the hypothesis that the former compound is a precursor of the latter. The two terminal P-atoms of ATP are known to contain most of the P^{32} activity of this compound and the phosphate attached to carbon atom 3 of 2,3-DPG has a very low activity as evidenced by measurements of the P^{32} activity in 3-phosphoglyceric acid (2). It thus seems reasonable to assume that the phosphate on carbon atom 2 of 2,3-DPG has a much higher P^{32} activity than the phosphate of

[4] It should be pointed out that Bartlett (14), using ion-exchange column chromatography, found that the RSA of 2,3-DPG was lower than that of ATP.

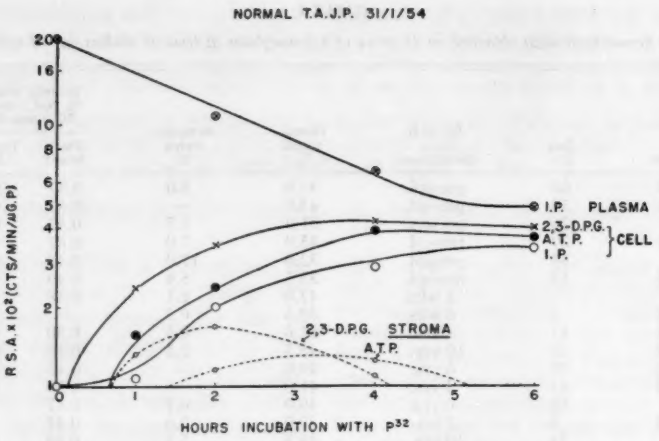


FIG. 1. RELATIONSHIPS OF RELATIVE SPECIFIC ACTIVITIES (RSA) OF PHOSPHATE COMPOUNDS IN NORMAL RED CELLS AFTER INCUBATION WITH P^{32}-PHOSPHATE

These curves are representative of the results obtained with blood samples from eight normal persons.

carbon atom 3 and that the highly active phosphate of 2,3-DPG is a precursor of the two terminal carbon atoms of ATP.

Studies on HS cells

The relationships of the intracellular P^{32}-phosphate fractions are altered in red cells from all of the HS patients studied (Figure 2; Table II), although the rate constant for P^{32} exchange remains within normal limits ($K = 0.22 \pm 0.02$ hr.$^{-1}$). The difference in P^{32}-phosphate partition manifests itself in most cases in a lower than normal RSA of ATP together with a less marked lowering in the RSA of 2,3-DPG and a higher than normal RSA of IP. In evaluating the data presented in Table II attention should be focused

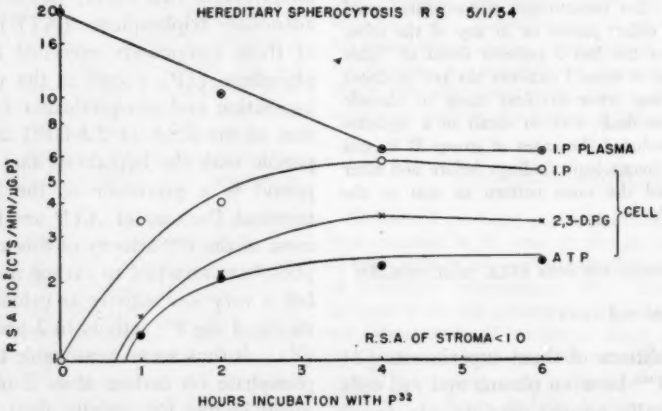


FIG. 2. RELATIONSHIPS OF RSA OF PHOSPHATE COMPOUNDS IN HS RED CELLS AFTER INCUBATION WITH P^{32}-PHOSPHATE

These curves are representative of the results obtained with blood samples from most of the patients studied.

TABLE II

RSA of various phosphate-containing compounds in red cells from patients with spherocytosis *

Case No.	After 2 hr. incubation				After 4 hr. incubation				
	Intracellular				Intracellular				Stromal
	2,3-DPG	ATP	IP	ADP	2,3-DPG	ATP	IP	ADP	ATP
A 1†	299	130	339	110	413	180	460	106	45
	(310)	(230)	(270)	(—)	(390)	(366)	(356)	(140)	
†	250	90	375	74	309	125	454	52	
A 2†	210	197	415	109	390	246	675	194	78
	(256)	(230)	(240)	(120)	(457)	(290)	(394)	(211)	
A 3†	260	139	325	112	400	203	515	177	100
	(270)	(240)	(225)	(96)	(416)	(410)	(400)	(134)	
A 4†	198	154	470	96	333	240	490	124	123
	(169)	(101)	(440)	(24)	(104)	(56)	(540)	(50)	
†	136	116	295	36	272	195	528	109	109
	(121)	(100)	(440)	(88)	(244)	(228)	(505)	(147)	
A 5†	200	140	190	52	280‡	140‡	310‡	60‡	73
	(230)	(108)	(150)	(60)	(300)	(175)	(300)	(100)	
A 6†	159	170	223	136	284	165	310	114	33
	(254)	(176)	(171)	(94)	(320)	(285)	(225)	(150)	
†	104	101	219	68	200	143	690	109	27
	(196)	(179)	(129)	(85)	(262)	(259)	(207)	(110)	
	120	155	160	120	190	135	220	150	54
	(112)	(185)	(100)	(90)	(195)	(195)	(100)	(96)	
A 7	106	100	148	58	198	176	248	160	71
	(124)	(70)	(54)	(64)	(234)	(118)	(179)	(84)	
A 8	234	200	323	70	465	150	580	—	60
	(280)	(228)	(267)	(110)	(425)	(350)	(317)	(180)	
	188	120	282	45	366	215	404	52	84
	(257)	(245)	(382)	(89)	(415)	(226)	(205)	(110)	
A 9	231	175	386	109	241	230	298	151	46
	(219)	(210)	(200)	(143)	(360)	(234)	(250)	(90)	
A 10	189	108	298	40	293	75	306	115	93
	(197)	(154)	(136)	(73)	(310)	(274)	(250)	(105)	
A 11	365	250	327	73	520	270	670	200	
	(453)	(190)	(450)	(52)	(515)	(323)	(420)	(190)	
A 12	209	185	235	157	375	300	400	250	
	(225)	(151)	(240)	(160)	(400)	(287)	(400)	(241)	
A 13	254	202	241	125	376	297	354	141	
	(221)	(175)	(289)	(141)	(354)	(241)	(360)	(162)	
A 14	250	200	220	90	380	300	400	100	94
	(240)	(250)	(180)	(100)	(390)	(275)	(300)	(80)	
A 15	148	131	354	44	168	140	405	66	21
	(215)	(184)	(153)	(100)	(310)	(255)	(168)	(115)	
	143	108	290	66	196	141	400	—	54
	(219)	(119)	(300)	(15)	(212)	(140)	(395)	—	
B 1	246	305	520	150	356	220	512	—	90
	(300)	(150)	(440)	(96)	(291)	(209)	(490)	(90)	
B 2	163	176	310	125	266	212	445	177	39
	(398)	(193)	(113)	(158)	(437)	(299)	(373)	—	
B 3	172	90	350	20	420	310	500	88	
	(200)	(155)	(226)	(150)	(340)	(200)	(340)	(125)	
Normals	287	236	239	110	404	336	348	151	125
8 persons tested	±34	±38	±36	±10	±20	±21	±41	±13	±30
	(295)	(220)	(236)	(114)	(420)	(319)	(359)	(134)	
	±15	±27	±32	±10	±21	±21	±36	±16	

* All figures in parentheses were obtained with red cells to which adenosine had been added.
† Pre-splenectomy.
‡ Partial clotting of the blood in this specimen after the second hour may have contributed to the low values of RSA of all fractions.

TABLE III

Mean concentrations of phosphate compounds in red cells from 8 normal persons and 14 persons with spherocytosis †*

	Normal	Spherocytosis
ATP	43.2 ± 5.4	39.2 ± 6.6
ADP	17.0 ± 4.1	25.9 ± 4.1
2,3-DPG	166.0 ± 10.1	156.1 ± 10.4
IP	59.4 ± 9.2	43.3 ± 6.0

* Excluding cases A 5, A 12, A 13, A 14.
† All figures represent μg. of P per ml. of packed red cells (mean ± S.D.).

primarily on the abnormal *relationships* between the RSA of the P^{32}-labeled compounds rather than on *differences in the absolute values* of RSA. The absolute values vary as might be expected from a heterogeneous red cell population such as exists in HS cases. The degree of heterogeneity of the red cells in this disease can be expected to vary from patient to patient, and the degree of metabolic abnormality in samples of HS blood can also be expected to vary considerably.

Analysis of the total quantity of the phosphate compounds present in these abnormal cells reveals nearly normal concentrations of ATP and 2,3-DPG, slightly higher concentration of adenosine diphosphate (ADP) and slightly lower concentration of IP (Table III).

The addition of adenosine to the incubating system of red cells did not alter intracellular P^{32} partition in normal cells (1–3), but in cells from 10 of the 18 patients investigated, it led to a shift of intracellular relationships towards normal. That is, the RSA of ATP was increased and that of IP reduced (figures in parentheses in Table II). It is of interest that the red cells of all affected members of each family thus far investigated either exhibited the described effect of adenosine on the partitioning of P^{32} or did not. These findings suggest that the response of the red cells to adenosine may be a genetically determined characteristic by which HS cases could be subclassified.

It has been mentioned in an earlier report (1) that 2,3-DPG, ATP, and ADP can be isolated from the stroma of red cells and that the RSA of these esters do not parallel those found intracellularly. The P^{32} activities of the ATP and 2,3-DPG fractions separated from the stroma of HS cells were so much lower than normal in many of the cases that accuracy of the radioactivity measurements was not as great as that of intracellular radioactivity. Since a deficiency in regeneration of ATP in the stroma or an increased activity of stromal ATP-ase might be related to the abnormal shape of HS red cells, an attempt was made to correlate the activities of the ATP fraction of HS stroma with the degree of sphering of the red cells in each case. Accurate estimates of the thickness-diameter ratios of the cells would have been helpful indicators of the degree of sphering (15). Lacking such data, we have taken the concentration of sodium chloride causing 50 per cent hemolysis of freshly drawn and incubated HS cells as a rough index of the degree of sphering (Table I). We have found, however, that more data will be required to show whether or not there is a close correlation between red cell fragility (or shape) and the ATP activity of the stroma.

DISCUSSION

Abnormalities of P^{32} partitioning in HS cells

Measurements of incorporation into ATP of P^{32} derived from extracellular P^{32}-orthophosphate suggest that in the red cells of patients with HS there are abnormalities in the dynamics of intracellular carbohydrate metabolism. Firstly, there is a smaller flux of P^{32} into ATP of the cell. Because of a nearly normal intracellular pool of ATP (Table III) it is concluded that the rate of P^{32} incorporation into ATP is slower. This may be associated with the lower potassium content of these cells (9, 16). The cation K^+ activates one of the phosphoferases, pyruvic phosphoferase, which phosphorylates ADP by transferring phosphate from phosphopyruvate to ADP. Secondly, the RSA of orthophosphate is elevated considerably and exceeds that of 2,3-DPG and ATP, in contrast with normal cells.

Although under normal conditions intracellular orthophosphate probably arises largely from the breakdown of ATP, this appears not to be true for the HS cell because the RSA of IP exceeds that of ATP. In view of the data in Table III, it seems probable that this change in the relationship of the RSA of IP to the RSA of ATP, as well as to that of 2,3-DPG, is due to changes in

the rate of turnover of phosphate rather than to changes in the size of the respective pools. It seems possible that an increased rate of exchange of plasma P^{32} with intracellular P^{32}, without change in the P^{32} exchange constant, might explain the altered P^{32} partition. This could be brought about if a greater proportion of intracellular P^{32} orthophosphate were incorporated into phosphate esters as compared with the proportion of extracellular P^{32} orthophosphates on the cell surface. It is also conceivable that a compound having an RSA higher than that of ATP or 2,3-DPG contributes at a greater than normal rate to the intracellular orthophosphate pool. One such compound could be 1,3-DPG from which orthophosphate may be liberated by phosphatase action. There may well be other as yet unknown sources of orthophosphate.

Effect of adenosine on phosphate metabolism of HS cells

The effect of adenosine in restoring towards normal the intracellular P^{32} partition may be attributed in part to the conservation of ATP since phosphorylative cleavage of adenosine resulting in the formation of ribose phosphate does not require ATP, as does the phosphorylation of glucose (17). The ribose phosphate(s) thus formed can be metabolized further to yield triose phosphate and hexose phosphates. It is, therefore, possible that adenosine increases the flux of P^{32} into the intracellular phosphate-containing compounds of HS cells by providing increased amounts of substrates which may serve as phosphate acceptors or may result in an increased rate of regeneration of ATP. Regeneration of ATP is in part dependent upon an adequate supply of ADP. This must be taken into account in formulating any postulate for the mechanism of action of adenosine on HS cells.

One alternative mechanism which remains as yet without direct supporting evidence may be concerned with the formation of adenylic acid from adenosine, leaving intact the N-riboside linkage of adenosine. Adenylic acid might then give rise to ADP and ATP by the action of an adenylate kinase.

Adenosine may well perform its function at the cell surface where phosphorylative cleavage could occur since the required phosphorylase has been found in the stromal fraction (18) and since the available evidence indicates that adenosine does not enter the red cell.

The P^{32} activity of ATP was not decreased as significantly in some cases, especially A 12, A 13, and A 14, as in the other cases but the abnormal relationship of P^{32}-labeled substances was nevertheless demonstrated. The red cells from 7 of the patients in group A (A 4 and A 7 from one family and A 5, A 12, A 13, and A 14 from another and A 15 from still another) and from one in group B (B 1) did not exhibit the usual response to incubation with adenosine. The apparent inability of adenosine to shift the intracellular distribution of P^{32} toward normal in some cases suggests that the necessary phosphorylase may be deficient in the cell stroma although other obvious possibilities exist. This enzyme has recently been demonstrated in stromal extracts of normal red cells following electrophoresis (18). In view of the uniformity of response to adenosine on the part of red cells from all afflicted members of each family studied, it is tempting to postulate that a genetically determined enzyme deficiency or functional inadequacy may be related to the lack of response to adenosine noted in members of certain families. Further genetic, hematologic, and biochemical studies will be needed before the significance of such irregularities can be understood. Such investigations may provide a basis upon which the various types of chronic spherocytosis can be more precisely classified. Somewhat similar changes have been found in the distribution of P^{32} in the cells from a number of different acquired hemolytic disorders, but only in HS has the corrective effect of adenosine been observed.

Relationship of metabolic abnormalities of HS cells to hematologic findings

The low P^{32} activity found in the stromal ATP fraction of HS cells may throw some light on the mechanism of sphering. Our findings suggest that an adequate rate of regeneration of ATP, and thus an adequate availability of energy-rich phosphate bonds, is necessary for the maintenance

of the biconcave shape. This may involve the function of certain contractile elements in red cell stroma. As we have suggested elsewhere (2), such a contractile element may resemble myosin, and like myosin might have ATP-ase activity. The demonstration of ATP-ase in red cell stroma (19) supports this view.

It seems likely that the biochemical abnormalities of HS cells are related to the principal hematologic findings and to the role of the spleen in this disease. When sterile defibrinated blood from HS patients is incubated at body temperature for 24 hours, the red cells undergo more rapid sphering and greater increase in osmotic fragility than do normal red cells (8, 20, 21). HS cells incubated similarly for 48 hours hemolyze much more readily than normal red cells, but if glucose, mannose, adenosine, or guanosine is added to the HS blood before incubation autohemolysis is markedly reduced (9, 21). Some details of these changes have been reported by Selwyn and Dacie (9). During the first 24 hours the cells swell because of acquisition of sodium and water, but during the second 24 hours they lose potassium, shrink, and finally hemolyze. In the opinion of these authors hemolysis during incubation is the result of a degeneration of the cell membrane. Similar changes have been observed in this laboratory. The rapid loss of potassium observed might be related to the high rate of sodium extrusion from HS cells reported by Harris and Prankerd (22). A normal rate of disappearance of glucose in incubated HS blood has been reported by Selwyn and Dacie (9) and has also been demonstrated repeatedly in this laboratory. To our knowledge, however, there are no reported studies on the rate of lactic acid formation in incubated HS blood.

Measurements of spontaneous lysis occurring in incubated blood reveal greater susceptibility to hemolysis of the HS cell with any depletion in the supply of metabolites available for energy production. The various substances tested may afford this cell protection by enabling glycolysis to continue and the potential energy within the cell to be maintained. The channels through which this energy is ultimately directed are multiple and include the control of electro-chemical ionic gradients, maintenance of structure and turnover of lipids of the cell membrane, and possibly other functions, the failure of any of which may result in hemolysis.

Relationship of metabolic abnormalities of HS cells to the mechanism of red cell destruction in vivo

The changes occurring on *in vitro* incubation of HS cells may be related to the effects of stagnation and hemoconcentration in the spleen, as first suggested in 1940 by Ham and Castle (23). Selective trapping of HS cells in the spleen has since been demonstrated (13, 20), and Gibson and associates (24) have shown that the hematocrit of splenic blood may be as high as 80 per cent. We can therefore postulate that the supply of substrate per cell would be reduced within the spleen, and would be still further depleted by the metabolism of the mass of lymphoid tissue in the splenic pulp. The HS cells trapped within the spleen might then be faced with "relative starvation," a situation similar to that obtaining in the previously cited *in vitro* experiments. After the splenic trap is removed these cells may survive normally because of continuous access to plasma constituents, despite persistence of the metabolic and structural abnormalities.

SUMMARY

1. A defect in intracellular glycolysis involving a smaller flux of P^{32} into ATP and 2,3-DPG with a concurrent increase in the flux into orthophosphate was observed in cells from patients with hereditary spherocytosis. The turnover of P^{32} of ATP and 2,3-DPG in the stromal fraction tended to be lower than normally found.

2. These abnormalities persist after splenectomy and in most cases were found to be largely restored to normal on incubation of the cells with adenosine. Lack of the corrective effect of adenosine in some cases suggests that the group of patients, now regarded as cases of hereditary spherocytosis, may not be homogeneous.

3. The abnormalities in carbohydrate metabolism have been correlated with the clinical course of the disease, and it is suggested that the susceptibility of the red cells to hemolysis may be due to an inadequate supply or utilization of energy within the cells or stroma in the form of high energy phosphate bonds.

Addendum

Recent experiments in this laboratory have revealed that a number of the compounds participating in the oxidative glucose-6-phosphate shunt are labeled by P^{32} under the experimental conditions described in this paper. It has also been ascertained that HS cells from those patients not responding to adenosine are not deficient in purine N-riboside phosphorylase.

The formation of adenylic acid from extracellular adenosine can now be excluded as an explanation of the effect of adenosine described in this paper, since one of us (T.A.J.P.) has found that adenine, resulting from phosphorolysis of adenosine added to the plasma, does not enter the cell.

Since this manuscript was prepared, we have learned that other workers have found ATP-ase in stroma:

a) Clarkson, E. M., and Maizels, M., Distribution of phosphatases in human erythrocytes. J. Physiol., 1952, **116**, 112.

b) Herbert, E., A study of the liberation of orthophosphate from adenosine triphosphate by the stromata of human erythrocytes. Ph.D. Thesis, University of Pennsylvania, 1952.

ACKNOWLEDGMENT

We wish to thank Mrs. Marion Murphy, Mrs. Pearl S. Miller, and Miss Mary Jane Izzo for their technical assistance.

REFERENCES

1. Prankerd, T. A. J., and Altman, K. I., Phosphate partition and turnover in human red cells. Nature, 1954, **173**, 870.
2. Prankerd, T. A. J., and Altman, K. I., A study of the metabolism of phosphorus in mammalian red cells. Biochem. J., 1954, **58**, 622.
3. Prankerd, T. A. J., and Altman, K. I., The effect of adenosine on the phosphate exchange in mammalian red blood cells. Biochem. et Biophys. Acta, 1954, **15**, 158.
4. Caldwell, P. C., The separation of the phosphate esters of muscle by paper chromatography. Biochem. J., 1953, **55**, 458.
5. Wintrobe, M. M., Clinical Hematology, Third Edition, Philadelphia, Lea and Febiger, 1951, p. 303.
6. Chaplin, H., Jr., and Mollison, P. L., Correction for plasma trapped in the red cell column of the hematocrit. Blood, 1952, **7**, 1227.
7. Furth, F. W., Unpublished observations.
8. Young, L. E., Izzo, M. J., and Platzer, R. F., Hereditary spherocytosis. I. Clinical, hematologic and genetic features in 28 cases, with particular reference to the osmotic and mechanical fragility of incubated erythrocytes. Blood, 1951, **6**, 1073.
9. Selwyn, J. G., and Dacie, J. V., Autohemolysis and other changes resulting from the incubation in vitro of red cells from patients with congenital hemolytic anemia. Blood, 1954, **9**, 414.
10. Evans, R. S., and Duane, R. T., Acquired hemolytic anemia. I. The relation of erythrocyte antibody production to activity of the disease. II. The significance of thrombocytopenia and leukopenia. Blood, 1949, **4**, 1196.
11. Thompson, W. P., The splenic lesion in hemolytic jaundice. Bull. Johns Hopkins Hosp., 1932, **51**, 365.
12. Dacie, J. V., Familial haemolytic anaemia (acholuric jaundice), with particular reference to changes in fragility produced by splenectomy. Quart. J. Med., 1943, **12**, 101.
13. Young, L. E., Platzer, R. F., Ervin, D. M., and Izzo, M. J., Hereditary spherocytosis. II. Observations on the role of the spleen. Blood, 1951, **6**, 1099.
14. Bartlett, G. R., Personal communication.
15. Haden, R. L., The volume thickness index of the erythrocyte of man. J. Lab. & Clin. Med., 1935, **20**, 567.
16. Maizels, M., The anion and cation contents of normal and anaemic bloods. Biochem. J., 1936, **30**, 821.
17. Dische, Z., Synthesis of hexosemono- and diphosphate from adenosine and ribose-5-phosphate in human blood *in* Symposium on Phosphorus Metabolism. Baltimore, Johns Hopkins Press, 1951.
18. Prankerd, T. A. J., and Altman, K. L., Riboside phosphorylase in human red cell stroma. To be published.
19. Garzó, T., Ullmann, A., and Straub, F. B., Die Adenosintriphosphatase der roten Blutkörperchen. Acta physiol. Hung., 1952, **3**, 513.
20. Emerson, C. P., Jr., Shen, S. C., Ham, T. H., and Castle, W. B., The mechanism of blood destruction in congenital hemolytic jaundice. J. Clin. Invest., 1947, **26**, 1180.
21. Izzo, M. J., Altman, K. I., Swisher, S. N., and Young, L. E., Unpublished observations.
22. Harris, E. J., and Prankerd, T. A. J., The rate of sodium extrusion from human erythrocytes. J. Physiol., 1953, **121**, 470.
23. Ham, T. H., and Castle, W. B., Relation of increased hypotonic fragility and of erythrostasis to the mechanism of hemolysis in certain anemias. Tr. A. Am. Physicians, 1940, **55**, 127.
24. Gibson, J. G., 2nd., Seligman, A. M., Peacock, W. C., Aub, J. C., Fine, J., and Evans, R. D., The distribution of red cells and plasma in large and minute vessels of the normal dog, determined by radioactive isotopes of iron and iodine. J. Clin. Invest., 1946, **25**, 848.

THE EFFECT OF ACUTE RESPIRATORY ACIDOSIS ON THE INTERNAL EQUILIBRIUM OF POTASSIUM [1]

By BELDING H. SCRIBNER, KENNETH FREMONT-SMITH, AND JAMES M. BURNELL

(From the Veterans Administration Hospital, Seattle, and the Department of Medicine, University of Washington, Seattle, Wash.)

(Submitted for publication January 18, 1955; accepted April 13, 1955)

There are conflicting reports on the effect of acid-base imbalance on the intracellular-extracellular distribution of potassium. The *in vitro* studies of Fenn and Cobb (1), and studies in dogs by Abrams, Lewis, and Bellet (2), Keating and his co-workers (3), and Pitts (4) suggest that metabolic acidosis results in a shift of potassium from the cells to the extracellular space, while metabolic alkalosis may lead to inward movement of potassium. In apparent contradiction, muscle analyses from Darrow's laboratory (5–8) demonstrate that muscle potassium is low in metabolic alkalosis and normal or slightly high in metabolic and respiratory acidosis. The findings of Darrow have been confirmed in metabolic alkalosis and acidosis by Cotlove, Holliday, Schwartz, and Wallace (9) and have led to the assumption that acidosis causes potassium to enter the cells and alkalosis causes potassium to leave the cells. These conflicting views may be due to a failure to separate the effect of acid-base imbalance on internal distribution of potassium from its effect on changes in total body potassium. This study attempts to separate these two effects by using inhalation of CO_2 to alter pH in dogs with ligated ureters and in dogs with intact ureters. This technique has the advantage of maintaining the total body potassium at a nearly constant level and at the same time altering the pH without inducing volume changes in the extracellular space as a result of infusions of acidifying and alkalinizing salts.

Respiratory acidosis resulted in a progressive rise over a four-hour period in the concentration of potassium in the extracellular space. This alteration in concentration ratio of potassium between the extracellular space and the cells persisted as long as the alteration in pH persisted.

[1] Supported by grants from Abbott Laboratories, North Chicago, Illinois, Washington State Fund for Biology and Medicine, and Washington State Heart Association.

MATERIAL AND METHODS

All experiments were performed on mongrel dogs lightly anesthetized with Nembutal®. Respiratory acidosis was induced by inhalation of 30 per cent CO_2 and 70 per cent O_2 through a tight-fitting intratracheal catheter. Ventilation was well maintained and arterial oxygen unsaturation did not occur. Serial blood specimens were taken at frequent intervals with blood replacement.

Respiratory acidosis was studied in ten dogs while ten additional anesthetized dogs served as controls. Five dogs from each group underwent bilateral ureteral ligation immediately prior to the experimental period. Experiments in dogs with respiratory acidosis and in control dogs lasted ten hours when the ureters were ligated and six hours when the ureters were intact. Changes in electrolyte balances and in plasma electrolyte levels were followed for the duration of the experiments. Accurate urine collections were insured by washing the bladder with water and air.

Distribution of an intravenous potassium load was studied in ten additional dogs with intact ureters. Five control dogs and five dogs breathing 30 per cent CO_2 and 70 per cent O_2 received the potassium load. Respiratory acidosis was induced immediately prior to the beginning of the infusion, which was administered with a constant rate infusion pump using a solution which contained 400 mEq. per L. KCl and 100 mEq. per L. $KHCO_3$. This composition was selected to minimize any alteration of acid-base balance which might result from the infusion. In these experiments loading continued until the electrocardiogram demonstrated potassium intoxication as evidenced by the disappearance of P waves or widening of the QRS complex. The infusion was then slowed until a near steady state was achieved at the toxic level. This level was maintained for one hour or more. The infusion was then stopped. The final specimens were obtained about one-half hour after the infusion was stopped and the balance period terminated at this point.

In all experiments loss of water due to increased respiratory activity of the dogs breathing 30 per cent CO_2 and 70 per cent O_2 was minimized by running the gas through a porous filtercandle which was immersed in water. Differences in muscular activity due to increased respiratory effort of the acidotic dogs were minimized by keeping the control dogs so lightly an-

TABLE I

Serial changes in plasma potassium and pH in control dogs and dogs with respiratory acidosis

Dog	Time in hours	0	1/6	2	4	6	8	10
				A. Ligated Ureters				
Control								
1	K	3.9		3.8	3.5	4.8		
	pH	7.31		7.32	7.30	7.26		
2	K	4.4		4.8	5.8	7.7	6.7	5.5
	pH	7.28		7.40	7.35	7.41	7.43	7.52
3	K	4.3		4.4	4.9	4.9	4.7	4.7
	pH	7.29		7.35	7.26	7.44	7.50	7.59
4	K	3.9		3.8	4.3	4.4	4.9	4.2
	pH	7.30		7.50	7.23	7.24	7.36	7.50
5	K	4.0		4.1	4.5	5.0	4.9	4.8
	pH	7.46		7.49	7.46	7.43	7.34	7.40
Mean:	K	4.1		4.2	4.6	5.4	5.3	4.8
Mean:	pH	7.33		7.41	7.32	7.36	7.41	7.50
Acidosis								
6	K	4.4		6.0		7.8	9.4	9.5
	pH	7.41		6.92		6.91	6.91	6.92
7	K	4.6	4.0	6.7	8.2	7.7	7.3	7.8
	pH	7.50	7.17	6.86	6.83	6.78	6.78	6.72
8	K	4.2	4.5	5.8	7.5	8.1	8.8	9.1
	pH	7.37	6.88	7.04	7.05	6.87	6.96	7.05
9	K	4.0	4.0	7.0	8.6	8.3	8.1	9.2
	pH	7.36	6.95	6.95	7.13	7.20	6.98	6.96
10	K	4.0	4.4	6.1	6.3	6.6	6.0	6.8
	pH	7.37	7.10	7.16	7.18	7.22	7.23	7.18
Mean:	K	4.2	4.2	6.3	7.7	7.7	7.9	8.5
Mean:	pH	7.40	7.03	6.99	7.05	7.00	6.97	6.97
				B. Intact Ureters				
Control								
11	K	3.6			3.6	4.1		
	pH	7.40			7.42	7.40		
12	K	3.9			4.0	4.2		
	pH	7.39			7.32	7.50		
13	K	4.0			3.8	3.8		
	pH	7.34			7.41	7.45		
14	K	3.9		3.5	3.8	3.9		
	pH	7.46		7.55	7.43	7.43		
15	K	4.4		4.3	4.3	4.3		
	pH	7.35		7.47	7.44	7.50		
Mean:	K	4.0		3.9	3.9	4.1		
Mean:	pH	7.39		7.51	7.40	7.46		
Acidosis								
16	K	4.2	4.6	6.5	6.6	6.7		
	pH	7.42	6.96	6.75	6.75	6.72		
17	K	4.2	4.3	5.4	5.6	6.0		
	pH	7.35	6.88	6.90	6.90	6.84		
18*	K	3.8	3.8	4.8	5.4	4.6		
	pH	7.46	7.06	7.03	6.97	6.95		
19	K	4.3	4.0	6.2	7.0	6.9		
	pH	7.45	6.85	6.90	6.86	6.82		
20*	K	3.6	4.0	5.7	5.9	5.7		
	pH	7.43	7.00	6.91	6.94	6.94		
21†	K	4.1		7.1	9.3			
	pH	7.36	6.78	6.75	6.74			
Mean:	K	4.0	4.0	5.7	6.1	6.0		
Mean:	pH	7.42	6.95	6.90	6.88	6.85		

* Fasted 48 hours prior to acidosis.
† Not included in means because of functional oliguria.

esthetized that there were vigorous involuntary leg movements during the period of study.

Arterial blood samples were collected anaerobically from an indwelling femoral cannula. Whole blood pH was determined within one minute at 38° C. using a Coleman pH meter with a glass electrode. Heparinized plasma was analyzed for sodium and potassium using a Baird internal standard flame photometer. Plasma chloride was determined by a mercury titration (10). Plasma bicarbonate was determined by a titration method (11) modified in that the final titration was carried to the original pH of each sample instead of pH 7.4. This modification insured that the true bicarbonate value was obtained in specimens taken during 30 per cent CO_2 inhalation. Whole blood glucose was determined by the method of Benedict (12).

Changes in the internal distribution of potassium were calculated as follows:

1) Final volume of the Extracellular Space

$$E_2 = \frac{E_1Cl_1 + bCl}{Cl_2}$$

where:

E_1 = 20 per cent body weight = Initial volume extracellular space
E_2 = Final volume extracellular space
Cl_1 = Initial plasma chloride level
Cl_2 = Final plasma chloride level
bCl = Chloride balance

2) Change in the Extracellular Potassium Content

$$KE_1 = E_1 \times K_1$$
$$KE_2 = E_2 \times K_2$$
$$\Delta KE = KE_2 - KE_1$$

where:

KE_1 = Initial content of potassium in the extracellular space
KE_2 = Final content of potassium in the extracellular space
K_1 = Initial plasma potassium level
K_2 = Final plasma potassium level

3) Change in the Intracellular Potassium Content

$$\Delta KI = bK - \Delta KE$$

where:

ΔKI = Change in the intracellular potassium content
ΔKE = Change in the extracellular potassium content
bK = Potassium balance

The potassium balance was not corrected for nitrogen. Chloride space calculations were not corrected for plasma water or Donnan effect because these corrections did not significantly alter the potassium transfer calculations.

Basing calculation of potassium transfer on changes in the chloride space weights the data slightly in favor of our hypothesis because the chloride space increases in some of the acidotic animals. However, calculation of the data assuming no change in the extracellular space does not significantly alter the differences observed between control and acidotic animals.

RESULTS

Effect of respiratory acidosis on potassium transfer in dogs receiving no potassium load

Table IA records the serial changes in plasma potassium and pH in dogs with ligated ureters during respiratory acidosis and in control dogs with ligated ureters. Table IB contains similar data for animals with intact ureters. Figures 1A and 1B are graphs of the average change in the plasma potassium levels in dogs with ligated

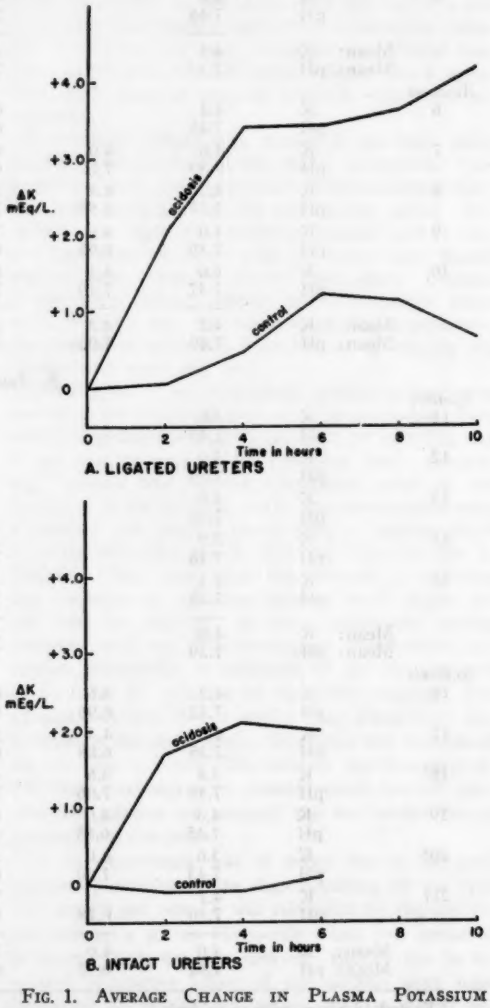


FIG. 1. AVERAGE CHANGE IN PLASMA POTASSIUM LEVELS IN CONTROL DOGS AND DOGS WITH RESPIRATORY ACIDOSIS

TABLE II

Data obtained in control dogs and dogs with respiratory acidosis

Dog	Wt. kg.	Exp. time hrs.	Blood: Hct. Init.*	Hct. 10 min.†	Hct. Final	Glucose Init.*	Glucose Max.	Glucose Time hrs.‡	Plasma: Bicarbonate Init.*	Bicarbonate Final	Potassium Init.*	Potassium Final	Chloride Init.*	Chloride Final	Sodium Init.*	Sodium Final	Urine: Vol. ml.§	Na^+ mEq.§	K^+ mEq.§	Cl^- mEq.§
											A. Ligated Ureters									
Control																				
1	15.0	6									3.9	4.8								
2	20.9	10	48		49	95	86	10	28.5	22.5	4.4	5.5	114	111	148	144				
3	18.6	10	49			90	75	4	24.8	24.0	4.3	4.7	110	108	147	145				
4	11.3	10	43		53				26.0	18.5	3.9	3.9	108	114	149	150				
5	14.9	10	62		58	128	210	4	25.0	18.0	4.0	4.8	110	111	149	147				
Acidosis																				
6	15.1	7									4.4	9.4								
7	10.7	10	45	66	67	99	204	¼	21.5	29.5	4.6	7.8	119	113	149	155				
8	22.7	10	51	61	67	172	210	¼	20.5	22.5	4.2	9.1	114	113	152	157				
9	21.0	10	63	55	54	141	232	4	23.8	29.5	4.0	9.2	115	111	148	150				
10	30.5	10	54	56	59	95	103	¼	17.5	24.8	4.0	6.9	107	105	148	157				
											B. Intact Ureters									
Control																				
11	13.5	6	41		41	99	89	3	21.3	22.5	3.6	4.1	110	110	150	149	38	2.2	8.6	2.8
12	15.5	6	39		40	85	104	2	17.5	22.0	3.9	4.2	109	109	146	145	54	6.8	11.9	6.7
13	11.8	6	38		41	86	66	5			4.0	3.8	112	113	147	145	30	4.4	5.6	5.9
14	21.3	6	36		39	79	87	4	27.0	23.0	3.9	3.9	112	111	146	139	295	27.4	16.2	12.8
15	18.5	6	40		50	88	63	1½	24.5	20.0	4.4	4.3	110	111	147	145	174	21.5	10.1	14.8
Acidosis																				
16	20.0	6							20.8	32.2	4.2	6.7	113	109	155	156	155	10.0	29.8	13.2
17	19.0	6	34	45	54	100	248	2	26.5	32.0	4.2	6.0	107	105	150	149	103	1.7	8.2	2.7
18‖	22.1	6	52	55	58	65	90	2	20.0	28.5	3.8	4.6	112	105	150	149	141	19.7	41.0	20.0
19	20.3	6	48	55	61	97	154	9	26.0	34.8	4.3	6.9	111	103	148	149	272	19.3	47.1	24.2
20‖	12.9	6	37	45	47	71	94	2	23.0	30.0	3.6	5.7	113	107	149	147	130	9.8	16.9	7.2
21	20.0	4	41		50				19.9	22.5	4.1	9.3	117	110	158	159	44	1.4	5.3	1.5

* Initial values obtained before induction of respiratory acidosis.
† Ten minutes after induction of respiratory acidosis.
‡ Time at which maximum glucose level was recorded.
§ Total amount excreted during experimental period.
‖ Fasted 48 hours prior to acidosis.

and intact ureters. In the acidotic dogs with ligated ureters plasma potassium levels rose an average of 4.3 mEq. per L. in ten hours as compared with 0.7 mEq. per L. rise in the controls. The plasma potassium level in acidotic dogs with intact ureters rose an average of 2.0 mEq. per L. in six hours as compared with a rise of 0.1 mEq. per L. in controls.

Tables IIA and IIB record the results of blood and urine analysis before beginning respiratory acidosis and during the experimental period. In addition to the potassium changes noted above, respiratory acidosis resulted in a rise in the plasma bicarbonate and a fall in the plasma chloride. Changes in the plasma sodium were generally small, although there was a slight rise in acidotic animals with ligated ureters. Blood glucose levels rose significantly in all but one of the non-fasted acidotic animals and tended to be maximal fifteen minutes after the onset of acidosis. Dogs No. 18 and No. 20, which were fasted for 48 hours prior to respiratory acidosis, demonstrated no elevation in blood glucose. The hematocrit rose sharply in all acidotic animals with a major portion of the rise complete within ten minutes. Urinary excretion of electrolytes varied widely and with the possible exception of dogs 18 and 19 which excreted a large amount of potassium during acidosis, there was no significant difference between control and acidotic dogs.

Calculated transfers of potassium from the cells to the extracellular space in dogs with ligated ureters are recorded in Table IIIA. The mean values in the last column of Table IIIA indicate that acidotic dogs with ligated ureters transferred approximately seven times as much potassium from the cells to the extracellular space as did the controls.

Calculated transfers in dogs with intact ureters are recorded in Table IIIB. The mean values in the last column indicate that acidotic dogs with intact ureters lost three times as much potassium from their cells as did the controls. This difference occurred despite a fairly large loss of potassium from the cells of the control dogs which resulted from basal renal excretion (daily turnover) of potassium. Even more significant was the increase of 0.41 mEq. potassium per kg. body weight in the extracellular space of acidotic dogs as compared with no change in the control dogs (column 4, Table IIIB).

In the one dog (No. 10) in which the plasma potassium level was followed after the termination of acidosis, the level fell from 6.8 mEq. per

TABLE III

Transfer of potassium in control dogs and in dogs with respiratory acidosis *

A. Ligated ureters				B. Intact ureters					
Dog	ΔCl space *liters*	ΔKe† *mEq.*	ΔKe† *mEq./kg.*	Dog	ΔCl space *liters*	ΔKe *mEq.*	ΔKe *mEq./kg.*	ΔKi *mEq.*	ΔKi *mEq./kg.*
Control				*Control*					
1	0.00	2.7	0.18	11	−0.02	1.3	0.10	− 9.9	−0.73
2	0.10	5.1	0.24	12	−0.06	0.7	0.05	−12.6	−0.81
3	0.06	1.8	0.10	13	−0.07	−0.8	−0.07	− 4.8	−0.41
4	−0.12	−0.5	−0.04	14	−0.09	−0.4	−0.02	−15.8	−0.74
5	−0.02	2.3	0.15	15	−0.17	−1.1	−0.06	− 9.0	−0.49
		Mean:	0.13			Mean:	0.00	Mean:	−0.64
Acidosis				*Acidosis*					
6	0.00	15.1	1.00	16	0.02	10.1	0.50	−39.9	−2.00
7	0.12	7.7	0.72	17	0.04	7.0	0.37	−15.3	−0.80
8	0.04	22.6	1.00	18‡	0.11	4.0	0.18	−45.0	−2.03
9	0.15	23.2	1.11	19	0.09	11.1	0.55	−58.2	−2.84
10	0.14	18.5	0.61	20‡	0.08	5.9	0.46	−22.8	−1.77
				21§	0.23	22.8	1.16	−28.1	−1.41
		Mean:	0.89			Mean:	0.41	Mean:	−1.89

* ΔKe—change in potassium in extracellular space.
ΔKi—change in potassium in intracellular space.
† ΔKe equals ΔKi because ureters are ligated.
‡ Fasted 48 hours prior to acidosis.
§ Not included in means because of functional oliguria.

TABLE IV

Effect of hypertonic potassium load on composition of blood and urine in control dogs and dogs with respiratory acidosis

Dog	Wt. *kg.*	Infusion			Blood pH Average	Plasma				Urine		
						Potassium		Chloride				
		Time *hours*	K^+ *mEq.*	Cl^- *mEq.*		Initial	Final*	Initial	Final*	Vol. *ml.*	K^+ *mEq.*	Cl^- *mEq.*
Control												
22	19.0	1.6	73	58	7.45	3.4	5.8	111	115	372	43.7	24.0
23	20.0	1.5	61	49	7.47	3.7	8.5	108	114	50	5.9	3.0
24	16.2	1.6	80	64	7.30	3.0	5.8	112	119	206	25.1	23.0
25	27.0	1.8	135	108	7.33	4.4	9.7	111	117	259	34.0	32.0
26	16.3	1.9	99	79	7.36	3.8	6.8	109	115	241	31.0	41.9
Acidosis												
27	16.4	3.6	38	30	6.90	3.5	8.8	107	102	406	19.6	29.5
28	17.6	1.3	43	35	6.82	3.9	10.4	112	110	356	18.5	7.9
29	8.5	1.8	31	25	7.01	3.8	8.4	108	109	220	25.0	42.5
30	11.0	2.2	17	14	6.89	3.9	10.4	115	114	2	0.2	0.1
31	14.8	2.8	39	31	6.99	4.3	7.9	115	110	226	32.2	26.9

* Level taken one-half hour after termination of infusion.

L. to 4.8 mEq. per L. in eight hours. This fall must represent a re-entry of potassium into the cells since ureteral ligation prevented urinary loss and there were no observed gastro-intestinal losses.

Distribution of a potassium load in dogs with respiratory acidosis

The data recorded in Table IV were used to calculate the distribution of potassium infused into five acidotic and five control dogs. Table V records the calculated transfers. It is important (column 3, Table V) that despite the large amount of chloride infused, the changes in the chloride space were small, similar in both control and acidotic dogs and therefore did not contribute to the differences observed in the distribution of retained potassium. The mean values recorded in the last column of Table V indicate that on the average only 27 per cent of the potassium retained by the control dogs remained in the extracellular space. In contrast, 113 per cent of the potassium retained by the acidotic dogs remained in the extracellular space. In other words, the cells of acidotic dogs lost potassium despite an

TABLE V

Distribution of infusion of potassium in control dogs and dogs with respiratory acidosis *

Dog	Balance		ΔChloride space *liters*	ΔKe *mEq.*	ΔKe *mEq./kg.*	ΔKi *mEq.*	ΔKi *mEq./kg.*	Retained K^+ remaining in extracellular space %
	K^+ *mEq.*	Cl^- *mEq.*						
Control								
22	29	34	0.17	10.0	0.53	19.0	1.00	33
23	55	46	0.19	20.8	1.04	34.2	1.71	38
24	55	41	0.14	9.9	0.61	45.1	2.79	18
25	101	76	0.38	32.2	1.19	68.8	2.54	32
26	68	37	0.14	10.7	0.66	57.3	3.51	16
							Mean:	27
Acidosis								
27	18	0	0.16	18.8	1.15	−0.8	−0.05	104
28	24	27	0.31	26.0	1.48	−2.0	−0.11	108
29	7	−18	−0.17	6.4	0.75	0.6	0.07	91
30	17	14	0.15	15.8	1.43	1.2	0.11	93
31	7	4	0.16	11.9	0.80	−4.9	−0.33	170
							Mean:	113

* ΔKe—Change in potassium in extracellular space.
ΔKi—Change in potassium in intracellular space.

external potassium load which elevated the plasma potassium to toxic levels. Since the longer loading period and slightly higher serum levels in the acidotic dogs would favor movement of potassium into the cells of the acidotic dogs, the differences observed are even more significant.

DISCUSSION

The confusion regarding the effect of acid-base imbalance on the internal distribution of potassium may be due to the fact that large changes in total body potassium have in the past obscured alterations in internal distribution of potassium. The above experiments with respiratory acidosis, in which changes in total body potassium were minimized, clearly demonstrate an increase in the concentration of potassium in the extracellular space of acidotic dogs. Regardless of the source of this potassium, the concentration ratio between the extracellular space and the cells $(K)_E/(K)_I$, was increased and the increase was maintained as long as the pH remained altered. The fact that infused potassium markedly elevated the serum level of acidotic dogs without causing transfer of potassium into the cells further supports the conclusion that acidosis raises $(K)_E/(K)_I$.

That $(K)_E/(K)_I$ is elevated in acidosis was first suggested by the observations of Fenn and Cobb in 1934 (1). Studying isolated frog muscle they noted that when the external media was acid, a higher than normal potassium level was required in the media to prevent potassium loss from muscle. Abrams, Lewis, and Bellet (2), infusing ammonium chloride rapidly into dogs, noted an inverse relation between pH and serum potassium level. Keating and his co-workers (3) performed experiments similar to those of Abrams, Lewis, and Bellet but used nephrectomized dogs whose body space volumes were accurately measured. Their data demonstrate that metabolic acidosis produced by sodium chloride or ammonium chloride infusions caused hyperkalemia as a result of a transfer of potassium from the cells to the extracellular space. Pitts (4) infused HCl into nephrectomized dogs and obtained results similar to those of other workers (3).

Our studies of respiratory acidosis by demonstrating the same increase in the concentration ratio of potassium as was demonstrated in metabolic acidosis by Fenn and Cobb (1), Abrams, Lewis, and Bellet (2), Keating and his co-workers (3), and Pitts (4) permit the conclusion that the increase in $(K)_E/(K)_I$ was related to pH change rather than to alteration in bicarbonate or pCO_2.

If acidosis raises $(K)_E/(K)_I$ then alkalosis may decrease the ratio. Fenn and Cobb (1) studying isolated frog muscle noted that when pH of the external media was elevated, a lower than normal potassium concentration still prevented potassium loss from the muscle. Keating and his co-workers (3) demonstrated that when sodium bicarbonate was infused into nephrectomized dogs there was a fall of about 1 mEq. per L. in the serum potassium level. The extracellular potassium content increased about 1 mEq. due to expansion of the extracellular space. Pitts (4) reported a typical experiment involving the infusion of sodium bicarbonate into a nephrectomized dog. He noted a decrease of 1.6 mEq. per L. in serum potassium level and a decrease of about 15 per cent in the potassium content of the extracellular space despite a marked increase in the volume of this space. Studies by Stanbury and Thomson (13) of acute respiratory alkalosis in humans demonstrated both a fall in serum potassium level and an increase in urinary excretion of potassium. Although the increase in potassium excretion may have accounted for the fall in the serum level, the fact that potassium did not shift from the cells to sustain the serum level suggests that the respiratory alkalosis may have lowered $(K)_E/(K)_I$ to prevent this shift. Respiratory alkalosis (pH 7.8) was studied in this laboratory using dogs with ligated ureters. Respiratory alkalosis invariably caused a fall in the serum potassium to levels as low as 2.6 mEq. per L. The average decrease in the serum potassium level was 2.2 mEq. per L. below control levels, and was sustained as long as the pH remained elevated (14).

The data in this paper when considered in conjunction with the earlier work on metabolic acidosis and metabolic and respiratory alkalosis permit the formulation of a hypothesis: acidosis increases and alkalosis decreases the potassium concentration ratio between the extracellular space and the cells. Expressed in another way: $(K)_E/(K)_I$ varies inversely with pH of plasma. In citing the above references on alkalosis in support of this hypothesis it is important to distin-

guish changes in extracellular potassium concentration from changes in extracellular potassium content. For example, in the experiments of Keating and his co-workers (3) potassium content in the extracellular space actually increased slightly due to expansion of the space when sodium bicarbonate was infused to produce alkalosis. However, the falling serum level halved $(K)_E/(K)_I$. Acidifying infusion of NaCl, on the other hand, while causing equal expansion of the extracellular space resulted in a rise in the serum potassium level which doubled this ratio.

Muscle analyses in humans by Mudge and Vislocky (15) suggest that alterations in the potassium concentration ratio induced by acid-base disorders also exist in states of chronic potassium depletion. Tables IA and IB of Mudge and Vislocky (15) record serum potassium levels in mEq. per L. and muscle potassium levels in mEq. per L. of intracellular water. $(K)_E/(K)_I$ may be calculated for each patient. With the exception of patient R-3 who was studied *post mortem*, $(K)_E/(K)_I$ is uniformly and significantly lower in alkalotic than in acidotic patients. It is important to point out that potassium depletion alone will also decrease $(K)_E/(K)_I$. (Potassium depletion causes a greater fall in the serum level than in the intracellular level.) In spite of this, the alkalotic patients had lower ratios than the acidotic patients who were more severely depleted of potassium. For example, patient R-1 with alkalosis (venous pH 7.53) had a serum potassium level of 2.2 mEq. per L. and a muscle potassium level of 136 mEq. per L. giving a $(K)_E/(K)_I$ of 0.016. Patient P with acidosis (venous pH 7.25) had a serum potassium level of 2.9 mEq. per L. and a muscle level of 120 mEq. per L. giving a ratio of 0.024. In other words, the patient with acidosis had a higher serum level even though he was more depleted. Patient K with severe alkalosis (venous pH 7.62) is of particular interest. He had a low serum potassium level (1.8 mEq. per L.) and a nearly normal muscle level (146 mEq. per L.) giving a ratio of 0.012. If intracellular potassium levels are expressed as mEq. per kg. fat free solids the differences in the ratios are even more striking. These studies of Mudge and Vislocky therefore support the hypothesis that $(K)_E/(K)_I$ varies inversely with plasma pH and suggest that the ratio remains altered as long as the plasma pH remains altered.

When the apparently conflicting data on muscle analyses from Darrow's laboratory (5, 6, 8) are reviewed in the light of the above discussion it is apparent, as the authors point out, that their finding of a low potassium level in muscle of rats with chronic metabolic alkalosis is the result of increased renal loss of potassium. This renal response has been confirmed in dogs by Franglen, McGarry, and Spencer (16). However, these muscle analysis experiments, including the study of chronic respiratory acidosis (7) cannot be used to support the concept that raising the extracellular pH causes an internal redistribution of potassium toward the extracellular space for the following reasons: First, a large change in total body potassium must have taken place in order to have measurable changes in muscle potassium. Second, calculation of $(K)_E/(K)_I$ for these experiments cannot be evaluated because the authors point out that serum potassium levels in rats anesthetized with ether are unreliable (6). Third, the experiments were such that at the time plasma and muscle measurements were made the pH levels were near normal.

Calculation of the ratio $(K)_E/(K)_I$ can be made from the data obtained in rats studied by Cotlove, Holliday, Schwartz, and Wallace (9). Although the pH alterations in acidosis and alkalosis, group II and III, were small acidosis increased and alkalosis decreased $(K)_E/(K)_I$. Group IV with potassium deficiency alone did not have the expected lowering $(K)_E/(K)_I$. Group V with potassium deficiency and acidosis had the same serum potassium level as alkalotic group III but a much lower muscle level. The studies of Cotlove with the exception of group IV, which the author emphasized was a non-homogenous group, are consistent with our hypothesis.

Discussion of the mechanisms by which pH change alters $(K)_E/(K)_I$ must be largely speculative. Fenn and Cobb (1) originally postulated a simple buffering mechanism between the cells and the extracellular space. They likened the potassium transfers to the chloride shift that takes place in the red blood cell with variations in pH. Since anions do not freely cross membranes of tissue cells, potassium and hydrogen shift during intracellular buffering instead of chloride and bi-

carbonate. Intracellular buffering may be the chief mechanism responsible for altering $(K)_E/(K)_I$ when the pH changes because acidosis and alkalosis alter the ratio in the opposite direction.

MacKay (17) has suggested glycogen breakdown to explain the hyperkalemia of respiratory acidosis. She produced respiratory acidosis in cats with 34 per cent carbon dioxide in oxygen and demonstrated a transient marked hyperkalemia. This was maximal at five minutes and returned to near normal at thirty to sixty minutes. Since this hyperkalemic response was largely abolished by evisceration, she concluded that it represented potassium released from liver glycogen. Respiratory acidosis in dogs is accompanied by hyperglycemia (Table II). However, glycogenolysis is not the principal source of the hyperkalemia for the following reasons: 1) Hyperglycemia was not maximal at the time of maximal hyperkalemia; 2) fasted dogs No. 18 and No. 20 developed hyperkalemia without hyperglycemia; 3) judging from the distribution of potassium loads in control dogs (Table V), 70 per cent of potassium released from glycogen would enter the cells if $(K)_E/(K)_I$ were unaltered by acidosis. This would require more potassium than exists in glycogen stores.

The possibility that acidosis may produce hyperkalemia through change in the partition of sodium must also be considered. A partial interference with the metabolic reactions supplying energy to the "sodium pump" might result in a shift of sodium into the cells and an outward shift of potassium during several hours of acute respiratory acidosis. The rising plasma sodium level in the acidotic dogs with ligated ureters suggests that, if anything, sodium entered the extracellular space. However, careful measurements of changes in the volume of the extracellular space are needed to determine the direction of the sodium transfer.

The rise in hematocrit values of acidotic dogs was largely complete within ten minutes and appears to be significant. It can be explained only in small part by the swelling of cells known to occur as part of the chloride shift. The other factors involved in this change in hematocrit await further investigation.

Even in the presence of intact kidneys (Figure IB) respiratory acidosis increased $(K)_E/(K)_I$. The data of Mudge and Vislocky (15) and of Cotlove, Holliday, Schwartz, and Wallace (9) demonstrate that in both alkalosis and acidosis the alterations in $(K)_E/(K)_I$ persist as long as the pH remains altered. These observations suggest that acidosis decreases and alkalosis increases renal potassium clearance. This is certainly true in alkalosis where concomitant increase in potassium excretion occurs.

The increase in the renal clearance of potassium in alkalosis may be explained by applying our hypothesis to the postulates of Berliner concerning potassium excretion. If, as Berliner, Kennedy, and Orloff propose (18), potassium excretion is a function of the concentration of potassium in the renal tubular cell, then alkalosis would be expected to increase renal potassium clearance by moving potassium into the renal tubule cell just as it may move it into all other cells.

The increased renal clearance of potassium in alkalosis explains the apparent paradox between our hypothesis and Darrow's observations. The fact that the muscle potassium becomes low in chronic alkalosis seems to conflict with the hypothesis that $(K)_E/(K)_I$ is lowered in alkalosis. However, the hypothesis defines a concentration ratio and does not preclude a concomitant decrease in total body potassium as a function of increased renal excretion. Hence, alkalosis leads to a decrease in the ratio $(K)_E/(K)_I$ as a result of movement of potassium into the cells, and a further small decrease in serum potassium induced by the kidneys in order that potassium be transported via the extracellular space from muscle to urine.

The fact that acid-base imbalance changes the serum potassium level by altering $(K)_E/(K)_I$ may prove to be of clinical importance. It has been pointed out that often the correlation is not good between the serum level and the total body potassium (19). This lack of correlation might be obviated in part if consideration were given to the increased $(K)_E/(K)_I$ in acidosis and the decreased $(K)_E/(K)_I$ in alkalosis. Thus hypokalemia in a patient with acidosis might indicate more severe potassium depletion than a similar degree of hypokalemia in a patient with alkalosis. This seems to be true in our limited experience and would be inferred from the studies of Mudge and Vislocky discussed above. That acute re-

spiratory acidosis may lead to fatal hyperkalemia under certain circumstances is a question of clinical importance. Observations on this subject have been reported (20, 21).

SUMMARY AND CONCLUSION

1. Acute respiratory acidosis in dogs causes hyperkalemia as a result of movement of potassium out of the cells.

2. When potassium is infused into dogs with respiratory acidosis virtually none of the administered potassium enters the cells. Similar infusions in normal dogs cause large transfers of potassium into the cells.

3. Accumulated evidence permits the formulation of the hypothesis that the internal equilibrium of potassium is in part a function of pH. Acidosis increases and alkalosis decreases the extracellular-intracellular concentration ratio of potassium. The altered ratio persists as long as the pH remains altered.

4. This hypothesis may be useful clinically in permitting better interpretation of the serum potassium level so that it more accurately reflects the potassium needs of the patient.

ACKNOWLEDGMENT

The authors wish to thank Dr. Clement A. Finch and Dr. Robert Evans for help in the preparation of the manuscript, and Miss Marjorie Ferguson, Miss Patricia Hoover, and Miss Elma Lile for technical assistance.

REFERENCES

1. Fenn, W. O., and Cobb, D. M., The potassium equilibrium in muscle. J. Gen. Physiol., 1934, **17**, 629.
2. Abrams, W. B., Lewis, D. W., and Bellet, S., The effect of acidosis and alkalosis on the plasma potassium concentration and the electrocardiogram of normal and potassium depleted dogs. Am. J. M. Sc., 1951, **222**, 506.
3. Keating, R. E., Weichselbaum, T. E., Alanis, M., Margraf, H. W., and Elman, R., The movement of potassium during experimental acidosis and alkalosis in the nephrectomized dog. Surg., Gynec. & Obst., 1953, **96**, 323.
4. Pitts, R. F., Mechanisms for stabilizing the alkaline reserves of the body. Harvey Lectures 1952–53, New York, Academic Press, 1954, p. 172.
5. Darrow, D. C., Changes in muscle composition in alkalosis. J. Clin. Invest., 1946, **25**, 324.
6. Darrow, D. C., Schwartz, R., Iannucci, J. F., and Coville, F., The relation of serum bicarbonate concentration to muscle composition. J. Clin. Invest., 1948, **27**, 198.
7. Cooke, R. E., Coughlin, F. R., Jr., and Segar, W. E., Muscle composition in respiratory acidosis. J. Clin. Invest., 1952, **31**, 1006.
8. Cooke, R. E., Segar, W. E., Reed, C., Etzwiler, D. D., Vita, M., Brusilow, S., and Darrow, D. C., The role of potassium in the prevention of alkalosis. Am. J. Med., 1954, **17**, 180.
9. Cotlove, E., Holliday, M. A., Schwartz, R., and Wallace, W. M., Effects of electrolyte depletion and acid-base disturbance on muscle cations. Am. J. Physiol., 1951, **167**, 665.
10. Scribner, B. H., Bedside determination of chloride: A method for plasma, urine, and other fluids and its application to fluid balance problems. Proc. Staff Meet., Mayo Clin., 1950, **25**, 209.
11. Scribner, B. H., and Caillouette, J. C., Improved method for the bedside determination of bicarbonate in serum. J.A.M.A., 1954, **155**, 644.
12. Benedict, S. R., Analysis of whole blood. II. The determination of sugar and of saccharoids (non fermentable copper-reducing substances). J. Biol. Chem., 1931, **92**, 141.
13. Stanbury, S. W., and Thomson, A. E., The renal response to respiratory alkalosis. Clin. Sc., 1952, **11**, 357.
14. Scribner, B. H., and Burnell, J. M., The effect of respiratory alterations of pH on the internal equilibrium of potassium. J. Clin. Invest., 1955, **34**, 919.
15. Mudge, G. H., and Vislocky, K., Electrolyte changes in human striated muscle in acidosis and alkalosis. J. Clin. Invest., 1949, **28**, 482.
16. Franglen, G. T., McGarry, E., and Spencer, A. G., Renal function and the excretion of potassium in acute alkalosis. J. Physiol., 1953, **121**, 35.
17. MacKay, J. L., Effects of a narcotic level of carbon dioxide on the plasma potassium and respiration of cats. Am. J. Physiol., 1947, **151**, 469.
18. Berliner, R. W., Kennedy, T. J., and Orloff, J., The relationship between potassium excretion and urine acidification *in* The Kidney, A. A. G. Lewis, ed., Boston, Little Brown & Company, 1954, pp. 147–164.
19. Moore, D. F., Edelman, I. S., Olney, J. M., James, A. H., Brooks, L., and Wilson, G. M., Body sodium and potassium. III. Inter-related trend in alimentary, renal and cardiovascular disease; lack of correlation between body stores and plasma concentration. Metabolism, 1954, **3**, 334.
20. Scribner, B. H., Bogardus, G. M., Fremont-Smith, K., and Burnell, J. M., Potassium intoxication during and immediately following respiratory acidosis. J. Clin. Invest., 1954, **33**, 965.
21. Young, W. G., Jr., Sealy, W. C., and Harris, J. S., The role of intracellular and extracellular electrolytes in the cardiac arrhythmias produced by prolonged hypercapnia. Surgery, 1954, **36**, 636.

ESTIMATION OF THE PHOSPHOLIPID PHOSPHORUS TURNOVER TIME IN MAN: STUDIES IN NORMAL INDIVIDUALS, IN PATIENTS WITH THE NEPHROTIC SYNDROME AND IN OTHER TYPES OF HYPERLIPEMIA[1]

By HUGO W. MOSER and KENDALL EMERSON, Jr.

(From the Department of Medicine, Harvard Medical School and the Medical Clinic, Peter Bent Brigham Hospital, Boston, Mass.)

(Submitted for publication December 17, 1954; accepted April 6, 1955)

The purpose of this study is to contribute to an understanding of the mechanism of production of various types of hyperlipemia by comparing the plasma phospholipid turnover time in normal individuals and in patients with elevated plasma lipids. The rate of incorporation of a single tracer dose of P^{32} labeled carrier-free inorganic phosphate into the plasma phospholipids has been measured and a mathematical procedure described by Zilversmit, Entenman, and Fishler (1) has been used for the calculation of the turnover time of the sum of phospholipid phosphorus contained in the plasma and liver. Results obtained in six normal individuals, in five patients with the nephrotic syndrome and in three patients with hyperlipemia due, respectively, to hypothyroidism, biliary cirrhosis and essential familial xanthomatosis will be presented.

MATERIALS

All studies were carried out at the Peter Bent Brigham Hospital. Experimental subjects were maintained on normal diet and activity. The subjects selected as normal controls were members of the house staff except for C. K. who was a ward patient. All were free from significant disease.

All patients with the nephrotic syndrome were considered to be in the nephrotic stage of chronic glomerulonephritis and none had azotemia. One of these patients, W. F., was studied on two occasions, marked, respectively, W. F. 1 and W. F. 2. Study W. F. 1 was carried out six weeks after onset of symptoms of the nephrotic syndrome, while study W. F. 2 was carried out three months later. In study W. F. 1, P^{32} was injected the day after cessation of a five-day course of oral cortisone, 300 mg. daily, which had failed to produce a diuresis. In addition, on the 4th, 5th, and 6th day following injection of the isotope the patient received 50 grams of albumin intravenously daily. The latter therapy, which produced a moderate diuresis, was accompanied by a fall in plasma phospholipid concentration from 18.7 to 13.4 mg. per 100 cc. Results of study W. F. 1 are thus difficult to interpret; they have been included in this series in order to avoid statistical bias. Studies in the other nephrotics were carried out six months to three years after onset of the disease. Except for study W. F. 1 all experiments in the patients with the nephrotic syndrome were carried out during periods in which the course of their disease was not changing.

Patient H. McC. presented the classical picture of hypothyroidism. Serum protein-bound iodine was 0.8 gamma per 100 cc., radioactive iodine uptake was 0 per cent and serum cholesterol was 480 mg. per 100 cc. Study No. 1 was carried out during a control period, study No. 2 performed after this patient had received 30 mg. of thyroid daily for three months. At this time there was marked clinical improvement and serum cholesterol had fallen to 300 mg. per 100 cc. Patient G. G. had suffered from pruritus, jaundice and progressive weakness for eight months. Physical examination showed intense jaundice, an enlarged, firm liver, palpable spleen tip but no xanthomata. Laboratory studies showed persistent elevation of serum alkaline phosphatase but only slight evidence of hepatocellular damage. Operation two months after onset of symptoms had failed to show extrahepatic biliary obstruction, while pathological findings in the liver were those of pericholangitis and early biliary cirrhosis.

Patient R. Go. presented the classical picture of familial hypercholesterolemia with xanthoma tendinosum, and there was well documented evidence of coronary insufficiency. Her serum cholesterol was 393 mg. per 100 cc.

METHODS

The radioactive phosphorus was obtained from the Oak Ridge National Laboratories as carrier-free P^{32} in the form of dilute phosphoric acid. The isotope was assayed and its identity checked qualitatively with aluminum absorbers. After a portion of the material had been set aside for half-life determination, it was diluted with physiological saline and autoclaved; 1.0 to 2.2 microcuries per kilogram of body weight were then injected intravenously into the experimental subjects, the smaller dosage being used in individuals who received more than

[1] This study was supported by a grant from the Department of Health, Education and Welfare, Public Health Service.

one injection. No untoward reactions were observed after a total of 26 injections in 22 subjects. In each experimental subject serial specific activity determinations were made of plasma lipid phosphorus and total urine phosphate one or more times daily for 5 to 7 days.

An end window tube Geiger counter with a counting efficiency for P^{32} of 25 to 30 per cent was used for radioactivity measurements. Background activity varied between 25 and 30 counts per minute. All samples were counted in the dry state, using disposable aluminum planchets. A minimum of 4,000 counts were obtained from each planchet. The radioactivity of all samples obtained later than 12 hours after injection of P^{32} was always more than five times and usually more than ten times background activity. Sample weight varied from 10 to 100 mg. A self absorption correction factor, determined experimentally in this laboratory, was applied to all samples. This correction factor did not exceed ten per cent.

Five to 10 cc. of heparinized plasma were used for lipid phosphorus specific activity determination. Plasma was separated within ten minutes of removal from the body and lipids were immediately extracted with a chloroform methanol mixture as described by Folch and his co-workers (2), the purification procedure in which the chloroform methanol extract is washed with a large volume of water was not found necessary. The chloroform methanol extract was then filtered and the filtrate was evaporated to dryness at reduced pressure, and at a temperature not exceeding 50 degrees centigrade. The residue was redissolved in about 15 cc. of chloroform and aliquots were taken for radioactivity determination and phosphorus analysis. The sample was digested with H_2SO_4 and H_2O_2 and the phosphorus determination was carried out according to the method of Fiske and Subbarow (3). The sample for radioactivity determination was placed in a centrifuge tube and its volume reduced to about 1 cc. by blowing a stream of nitrogen over its surface. This solution was transferred quantitatively to a planchet covered with a circle of lens paper and the chloroform was allowed to evaporate. The sample was then ready for counting. All determinations were performed in duplicate. The mean difference between 50 duplicate radioactivity determinations was 1.7 per cent with a standard deviation of 1 per cent. *In vitro* experiments had shown that this procedure allowed excellent separation of inorganic P^{32} from plasma lipid phosphorus. In these experiments only 0.1 to 0.2 per cent of inorganic P^{32} labeled phosphate added to nonradioactive plasma was present in the lipid extract.

Voided specimens were used for the determination of urine phosphate specific activity. Usually two-hour urine collections were obtained. For the specific activity of *total* urine phosphate, 1 cc. of urine was placed in a planchet, dried slowly under an infra-red lamp and counted. Phosphorus determination was carried out according to the method of Fiske and Subbarow (3). For the specific activity of urine *inorganic* phosphate the sample was treated with magnesia mixture (4), thus precipitating inorganic phosphate as magnesium ammonium phosphate. The washed precipitate was then dissolved in 0.1 N HCl and aliquots of this solution were taken for radioactivity determination and phosphorus analysis. Following precipitation of proteins with trichloracetic acid, the specific activity of *plasma inorganic* phosphate was measured in the same manner as that of urine inorganic phosphate.

The relationship between the specific activity of plasma inorganic phosphate, urine inorganic phosphate and total urine phosphate was studied in preliminary experiments. These experiments were prompted by the results of Handler and Cohn (5) and Govaerts (6) who found that in dogs subsequent to the first 30 to 90 minutes following injection of P^{32} the specific activity of plasma inorganic phosphate and urine inorganic phosphate are identical, and also by the fact that almost all urine phosphate is in the form of inorganic phosphate. In our experiments the specific activity of plasma inorganic phosphate was compared with that of urine inorganic phosphate in two normal subjects between 9 and 33 hours after injection of P^{32}. The average difference in five comparisons was 0.6 per cent with a maximum difference of 4 per cent. The specific activity of total urine phosphate and inorganic urine phosphate was compared in a total of twenty specimens from two normal individuals and two patients with the nephrotic syndrome. The average difference was 1.8 per cent with a standard deviation of 1.3 per cent. We have concluded, therefore, that subsequent to nine hours after injection of P^{32} and possibly before that time, the specific activity of total urine phosphate is equal to that of plasma inorganic phosphate. Use of urine, rather than plasma, to measure the specific activity of inorganic phosphate has made possible more frequent and accurate measurements.

RESULTS

The experimental data are shown in Table I and the results of certain experiments are presented in Figures 1–5. Specific activity is expressed as

$$\frac{\text{Counts per minute per gram of phosphorus}}{\text{Counts per minute injected per kg. of body weight}}$$

the denominator being introduced in order to make results obtained in different experiments more comparable. The most significant results have been plotted in Figures 3 to 5. It is shown that, in relation to the specific activity of urine phosphate, the specific activity of plasma lipid phosphorus increased more slowly than normal in a nephrotic and in a patient with hypothyroidism, while it appeared to increase at a normal rate in a patient with biliary cirrhosis. These relationships can be compared more quantitatively by calculating the phospholipid turnover time in each instance.

TABLE I

The specific activity of urine phosphate and plasma lipid phosphorus

Patient	Sex	Age	Diagnosis	Lipid* phosphorus Range mg. %		Specific activity† Hours after injection of P^{32} 12	24	36	48	60	72	96	120	144	168
P. A.	M	24	Normal	6.0–7.5	PO_4‡	4.38	2.90	2.28	1.85	1.58	1.47	1.25	1.13	1.02	0.942
					Lipid P	0.599	1.23	1.53	1.61	1.62	1.56	1.42	1.29	1.17	
O. B.	M	28	Normal	8.7–10.0	PO_4	4.13	2.85	2.03	1.72	1.46	1.30	1.12	1.01	0.924	0.847
					Lipid P	0.539	1.32	1.64	1.73	1.69	1.64	1.39	1.27		
A. S.	M	24	Normal	8.8–10.0	PO_4	3.87	2.39	1.97	1.69		1.36	1.19	1.06	0.963	
					Lipid P		1.36		1.59	1.53	1.44	1.28	1.11		
C. K.	M	29	Normal	8.2–9.0	PO_4		2.69		1.91	1.62	1.46	1.25	1.14		
					Lipid P		1.01	1.25	1.43	1.43	1.47	1.35	1.31		
R. C.	M	27	Normal	9.0–9.5	PO_4	2.76	2.15	1.80	1.58	1.41	1.28	1.12	1.05	1.00	0.956
					Lipid P	0.314	0.774	0.978	1.15	1.16	1.14	1.07	0.964		
F. H.	M	30	Normal	12.4–13.0	PO_4	3.48	2.49	2.09	1.87	1.71	1.57	1.42	1.32	1.24	1.17
					Lipid P		0.705		1.19	1.27	1.26	1.24			
W. F. 1	M	12	Nephrotic syndrome	13.4–20.1	PO_4		5.12	3.78	3.15	2.70	2.36	1.85	1.55	1.41	1.23
					Lipid P		1.03	1.76	2.25		2.32	2.39	2.17		
W. F. 2	M	12	Nephrotic syndrome	25.0–31.5	PO_4	7.45	4.51	3.60	3.08	2.69	2.48	2.08	1.89	1.75	1.62
					Lipid P		0.386		0.907		1.46	1.88	2.04		
A. G.	M	39	Nephrotic syndrome	12.1–13.7	PO_4		6.47	4.98	4.01	3.59	3.24	2.80	2.49	2.35	2.22
					Lipid P		1.11		2.04	2.20	2.44	2.48	2.44		
B. D.	M	19	Nephrotic syndrome	26.9–29.4	PO_4		6.34	4.37	3.50	2.99	2.64	2.16	1.87	1.63	
					Lipid P		0.764		1.63		2.05	2.09	2.04		
J. P.	M	17	Nephrotic syndrome	19.0–23.2	PO_4		4.71	3.38	2.56	2.10	1.82	1.46	1.24	1.09	0.959
					Lipid P		0.728	1.14	1.41		1.55	1.65	1.57	1.48	1.37
R. G.	M	16	Nephrotic syndrome	21.8–26.4	PO_4	7.71	4.73	3.30	2.62	2.20	1.95	1.65	1.45	1.28	1.12
					Lipid P		0.64	1.04	1.27		1.55	1.59	1.61	1.52	1.41
R. Go.	F	42	Familial hypercholesteremia	14.7–17.3	PO_4	4.48	3.13	2.37	1.98	1.69	1.48	1.25	1.16	1.09	1.01
					Lipid P		0.896		1.39		1.44	1.34	1.27		
G. G.	F	44	Biliary cirrhosis	45.0–53.1	PO_4		3.67	2.68	2.29	2.05	1.90	1.71	1.55	1.39	1.25
					Lipid P	0.548	1.35		1.89		1.94	1.95	1.78		1.59
H. McC. 1	F	62	Hypothyroidism untreated	18.5–20.2	PO_4		5.81	4.42	3.80	3.39	3.07	2.52	2.13	1.86	1.64
					Lipid P	0.232	0.689	1.14	1.46		2.02	2.12	2.22	2.17	2.08
H. McC. 2	F	62	Hypothyroidism treated	11.7–13.4	PO_4	7.51	5.06	3.80	3.13	2.73	2.45	2.09	1.89	1.69	1.50
					Lipid P	0.578	1.44	2.12	2.42	2.45	2.39	2.34	2.29	2.17	1.94

* "Lipid Phosphorus, Range mg. %" is the maximum and minimum concentration of plasma lipid phosphorus during the experiment.

† Specific activity is expressed as the ratio $\frac{\text{Counts per minute per gram of phosphorus}}{\text{Counts per minute injected per kg. of body weight}}$

‡ "PO_4" refers to the specific activity of total urine phosphate. For convenience of charting the urine phosphate specific activities shown here are not the experimental values, but have been taken from the curve which gave the best fit for all experimental values in any one patient. The extent to which experimental values deviated from these curves is shown in Figures 1, 2, and 7.

Calculation of turnover time

A mathematical treatment described by Zilversmit, Entenman, and Fishler (1) has been employed to calculate the turnover time of the sum of lipid phosphorus contained in the plasma and liver, henceforth referred to as the "plasma-liver pool" of lipid phosphorus. Turnover time is defined as the time required to turn over a quantity of phospholipid phosphorus equal to that present in the pool. It is known that there are several types of phospholipids each with different turnover rates (7, 8). Our data give information

THE SPECIFIC ACTIVITY OF URINE PHOSPHATE AND PLASMA LIPID PHOSPHORUS AFTER INJECTION OF P32

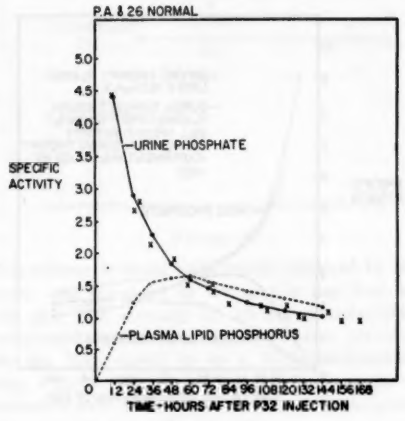


FIGURE 1

Specific activity in this and all other figures is expressed as:

$$\frac{\text{Counts per minute per gram of phosphorus}}{\text{Counts per minute expressed per kg. of body weight}}$$

The crosses indicate experimental values for urine phosphate specific activity. This is one of the experiments where Zilversmit's criteria for an immediate precursor were fulfilled (see text).

only about the average turnover time of these compounds and, furthermore, only about the phosphorus moiety of the molecule. This limitation will be discussed below.

The basic equation in this calculation is:

$$\frac{dL}{dt} = k(P - L), \qquad (1)$$

where

L = specific activity of lipid phosphorus,
P = specific activity of urine phosphate,
k = fraction of lipid phosphorus present in the "plasma-liver pool" turned over per hour,
t = time in hours.

Equation (*1*) is similar to a more general equation described by Zilversmit, Entenman, and Fishler (1). They also discussed certain general assumptions necessary for this calculation. To apply this equation to the data of the present study the following additional assumptions are required, due to the fact that the specific activity of

THE SPECIFIC ACTIVITY OF URINE PHOSPHATE AND PLASMA LIPID PHOSPORUS AFTER INJECTION OF P32

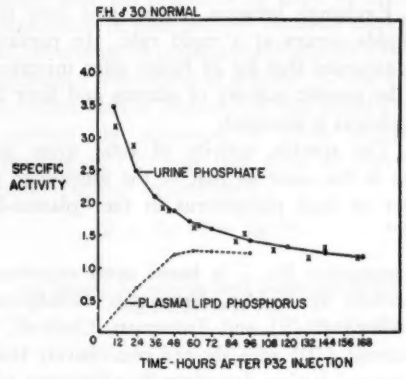


FIGURE 2

This is one of the two experiments where Zilversmit's criteria for an immediate precursor were not fulfilled (see text).

COMPARISON OF THE RATE OF INCREASE OF PLASMA LIPID PHOSPHORUS SPECIFIC ACTIVITY IN A NORMAL INDIVIDUAL AND IN A PATIENT WITH THE NEPHROTIC SYNDROME

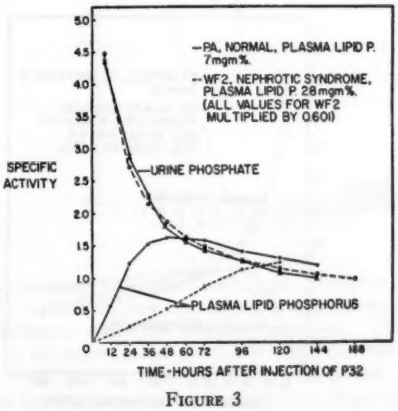

—PA, NORMAL, PLASMA LIPID P. 7mgm%.
--WF2, NEPHROTIC SYNDROME, PLASMA LIPID P 28 mgm%. (ALL VALUES FOR WF2 MULTIPLIED BY 0.601)
SPECIFIC ACTIVITY
URINE PHOSPHATE
PLASMA LIPID PHOSPHORUS
TIME-HOURS AFTER INJECTION OF P32

FIGURE 3

In preparing this figure all specific activity values for patient W. F. 2 were multiplied by 0.601, this factor being chosen because it made the urine phosphate specific activity curve for this patient and for the other individual shown in the figure almost superimposable. It is evident from the figure, that, in relation to the specific activity of urine phosphate, the specific activity of plasma lipid phosphorus increased much more slowly in the patient with nephrosis than in the normal individual.

liver inorganic phosphate and lipid phosphorus was not measured. It is assumed that:

1. All plasma phospholipids are both manufactured and destroyed by the liver.
2. Exchange between plasma and liver phospholipids occurs at a rapid rate. In particular, it is assumed that by 24 hours after injection of P^{32} the specific activity of plasma and liver lipid phosphorus is identical.
3. The specific activity of total urine phosphate is the same as that of the immediate precursor of lipid phosphorus in the "plasma-liver pool."

Assumption No. 1 is based upon experiments performed by Fishler, Entenman, Montgomery, and Chaikoff (9) and Entenman, Chaikoff, and Zilversmit (10) who showed conclusively that in the normal fasting dog virtually all plasma phospholipids are both manufactured in and removed from the circulation by the liver.

Assumption No. 2 is based upon experimental work in dogs by Fishler, Entenman, Montgomery, and Chaikoff (9) in which the specific activity of lipid phosphorus in the liver, plasma and certain other tissues was compared following the intramuscular injection of P^{32} labeled inorganic phosphate. It was found that by eighteen hours after injection of P^{32} and thereafter, plasma and liver phospholipid phosphorus had nearly identical specific activities. As will be discussed below this assumption may not be justified in the patients with nephrosis.

COMPARISON OF THE RATE OF INCREASE OF PLASMA LIPID PHOSPHORUS SPECIFIC ACTIVITY IN A NORMAL INDIVIDUAL AND IN A PATIENT WITH BILIARY CIRRHOSIS

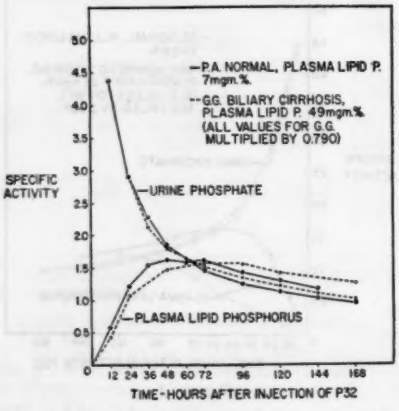

—P.A. NORMAL, PLASMA LIPID P. 7mgm.%.
--GG. BILIARY CIRRHOSIS, PLASMA LIPID P. 49mgm.% (ALL VALUES FOR G.G. MULTIPLIED BY 0.790)
SPECIFIC ACTIVITY
—URINE PHOSPHATE
—PLASMA LIPID PHOSPHORUS
0 12 24 36 48 60 72 96 120 144 168
TIME-HOURS AFTER INJECTION OF P32

FIGURE 4

The same procedure for charting was used as in Figure 3, except that in this instance all specific activity values for patient G. G. were multiplied by the factor 0.790. In spite of the markedly increased plasma phospholipid concentration in patient G. G., the specific activity of plasma lipid phosphorus increased at nearly the same rate as in the normal individual.

COMPARISON OF THE RATE OF INCREASE OF PLASMA LIPID PHOSPHORUS SPECIFIC ACTIVITY IN A PATIENT WITH HYPOTHYROIDISM BEFORE AND DURING THYROID THERAPY

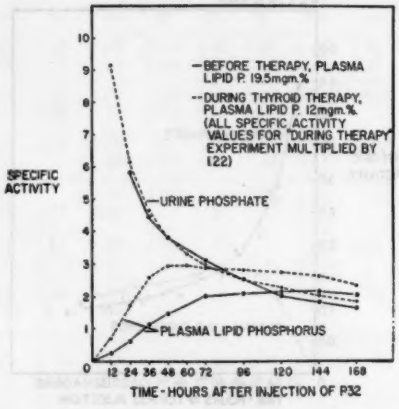

—BEFORE THERAPY, PLASMA LIPID P. 19.5mgm.%
--DURING THYROID THERAPY, PLASMA LIPID P. 12mgm.%. (ALL SPECIFIC ACTIVITY VALUES FOR "DURING THERAPY" EXPERIMENT MULTIPLIED BY 1.22)
SPECIFIC ACTIVITY
—URINE PHOSPHATE
—PLASMA LIPID PHOSPHORUS
0 12 24 36 48 60 72 96 120 144 168
TIME-HOURS AFTER INJECTION OF P32

FIGURE 5

The first study in patient H. McC. was carried out during a pre-treatment control period and the second study after therapy with thyroid extract 30 mg. a day for three months. In this figure all specific activity values for the "during therapy" study were multiplied by 1.22. It is evident from the figure that, in relation to the specific activity of urine phosphate, the specific activity of plasma lipid phosphorus increased more slowly before therapy than during therapy.

Assumption No. 3 has been evaluated by review of data available from experimental animals in regard to known intermediates between urine phosphate and the immediate precursor of liver phospholipids. The probable intermediate steps between urine phosphate and the immediate precursor of liver phospholipids are shown in Figure 6. As discussed earlier, equilibrium between the specific activity of plasma inorganic phosphate and urine phosphate is probably established rapidly.

In the rat the specific activity of plasma inor-

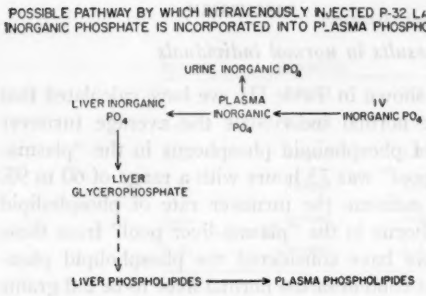

POSSIBLE PATHWAY BY WHICH INTRAVENOUSLY INJECTED P-32 LABELED INORGANIC PHOSPHATE IS INCORPORATED INTO PLASMA PHOSPHOLIPIDES
URINE INORGANIC PO4
LIVER INORGANIC PO4
PLASMA INORGANIC PO4
I.V. INORGANIC PO4
LIVER GLYCEROPHOSPHATE
LIVER PHOSPHOLIPIDES
PLASMA PHOSPHOLIPIDES

FIGURE 6

This scheme is based upon results obtained by several authors. As discussed in the text it has been shown in the dog that virtually all plasma phospholipids are manufactured in the liver (8, 9). Liver glycerophosphate has been shown to be a likely intermediate by several authors (8, 13, 15, 16). The pathway by which glycerophosphate is incorporated into phospholipids has not been established with certainty.

ganic phosphate and liver inorganic phosphate were found to be nearly the same subsequent to two hours after injection of P^{32} (11, 12). Popjak and Muir (13) and Zilversmit, Entenman, and Chaikoff (8), however, found that the specific activity of liver glycerophosphate is less than that of liver inorganic phosphate up to as long as 24 hours after injection of P^{32}. Zilversmit, Entenman, and Chaikoff (8) and Tolbert and Okey (14) showed in experimental animals that if calculations of turnover time of liver phospholipids are based upon the assumption that liver inorganic phosphate is the immediate precursor of liver phospholipids, resulting values are two to four times as long as when it is assumed that liver glycerophosphate is the immediate precursor. In these studies it was shown that liver inorganic phosphate clearly did not fulfill criteria set out by Zilversmit, Entenman, and Chaikoff and other workers (8, 13) for an immediate precursor of liver phospholipids. In our data, however, urine inorganic phosphate fulfilled the criteria for an immediate precursor of plasma phospholipid in eleven out of sixteen experiments in human subjects. In three of our studies (H. McC. 2, A. G., and G. G., Table I) the criteria were not entirely fulfilled, but the deviation was small and may have been due to experimental error, while in only two studies (R. C. and F. H., Table I and Figure 2) the immediate precursor relationship was clearly not fulfilled. These findings suggest that for studies in man, the assumption that inorganic phosphate is an immediate precursor of liver phospholipids does not introduce as large an error in the calculation of phospholipid turnover time as it did in the studies in experimental animals (8, 13).

Equation *1* was solved as follows. Using a procedure illustrated in Figure *7*, it was found that in all patients the variation of urine phosphate specific activity (P) with time could be expressed as the sum of two exponentials:

$$P = Ae^{-\lambda_1 t} + Be^{-\lambda_2 t}, \qquad (2)$$

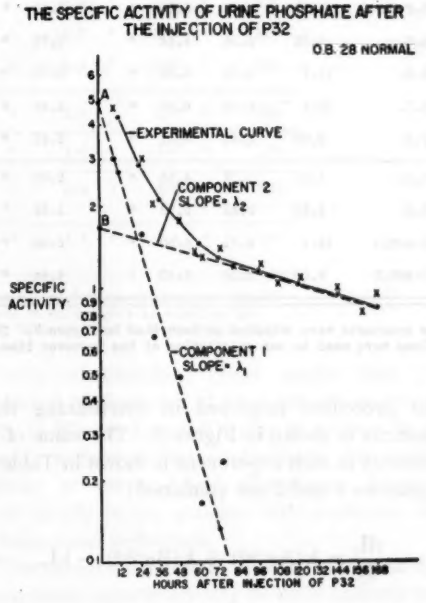


FIGURE 7

In this figure urine phosphate specific activity is plotted as the ordinate against time in hours on semi-log paper. Experimental points are indicated as crosses. The portion of the experimental curve between 96 and 168 hours was found to form a straight line. This portion of the curve was designated as component 2 with slope lamda 2, and was extrapolated to 0-time, as shown in the figure. The difference between the experimental curve and the extrapolated line at corresponding time intervals was determined graphically and plotted, these points being shown as open circles in the figure. Component 1, with slope lamda 1, is the straight line which gives the best fit for these points. Points A and B represent, respectively, the intercepts of components one and two with the 0 hour line. Using this procedure urine phosphate specific activity could be expressed by equation *2* (see text).

TABLE II

VALUES OF CONSTANTS FOR URINE CURVE USED FOR CALCULATION OF TURNOVER TIME

PATIENT	A	B	Lamda 1	Lamda 2
P.A.	3.87	1.66	4.32 x 10^{-2}	3.44 x 10^{-3}
O.B.	4.94	1.49	5.03 "	3.36 "
A.S.	5.07	1.86	7.35 "	4.52 "
C.K.	5.85	1.78	5.85 "	3.66 "
R.C.	2.43	1.33	3.98 "	1.94 "
F.H.	2.21	1.50	3.02 "	1.41 "
W.F.1	7.27	3.04	4.49 "	5.43 "
W.F.2	5.77	2.48	3.96 "	2.42 "
A.G.	9.02	3.52	4.44 "	2.75 "
B.D.	11.7	3.70	5.81 "	5.73 "
R.G.	10.2	2.78	6.28 "	5.45 "
J.P.	9.09	2.30	5.11 "	5.21 "
R.Go	5.22	1.57	4.53 "	2.60 "
G.G.	5.80	2.32	5.78 "	3.52 "
H.McC.1	15.1	4.71	8.50 "	6.48 "
H.McC.2	8.05	3.18	5.40 "	4.44 "

The constants were obtained as described in Figure 7. These values were used in our calculation of the Turnover Time.

The procedure employed in determining these constants is shown in Figure 7. The value of the constants in each experiment is shown in Table II. Equations *1* and *2* are combined:

$$\frac{dL}{dt} = k(Ae^{-\lambda_1 t}) + k(Be^{-\lambda_2 t}) - kL. \qquad (3)$$

Solution of equation *3* is:

$$L = \frac{kA}{k-\lambda_1} e^{-\lambda_1 t} + \frac{kB}{k-\lambda_2} e^{-\lambda_2 t} - \left(\frac{kA}{k-\lambda_1} + \frac{kB}{K-\lambda_2}\right) e^{-kt} \qquad (4)$$

"k" can be found numerically by substitution at any one time, "t" of the experimental values of L, A, B, lamda 1, and lamda 2. Results of this calculation in each patient at t = 24, 48, 72, and 96 are shown in Table III. Turnover time is equal to 1/k ("k" represents the fraction of lipid phosphorus turned over per hour).

DISCUSSION

A. *Results in normal individuals*

As shown in Table III, we have calculated that in six normal individuals the average turnover time of phospholipid phosphorus in the "plasma-liver pool" was 75 hours with a range of 60 to 95.

To estimate the turnover rate of phospholipid phosphorus in the "plasma-liver pool" from these data we have considered the phospholipid phosphorus content of the normal liver to be 2.0 grams (17), while we have used a figure of 0.3 gram for the phospholipid phosphorus content of plasma. The phospholipid phosphorus content of the "plasma-liver pool" would thus be estimated to be 2.3 grams. Turnover rate is equal to

$$\frac{\text{Lipid phosphorus content in "plasma-liver pool"}}{\text{Turnover time}}.$$

It is thus estimated that 2.3/75 or 0.03 gram of lipid phosphorus are turned over per hour.

Weinman, Chaikoff, Entenman, and Dauben (18) found that the turnover times of the fatty acid and the phosphorus moiety of the plasma phospholipid molecule were nearly the same, while Tolbert and Okey (14) showed that the phosphorus and choline moiety of rat liver phospholipids appeared to have the same turnover time. It is thus possible that the turnover time of the phospholipid molecule as a whole is the same as that of the phosphorus moiety. Using the conventional factor of 25 it would thus be estimated that 25 × .03 = 0.75 gram of phospholipid are turned over per hour or 18 grams per day. London and Rittenberg (19) calculated that the half-life time of plasma cholesterol in normal man was eight days. Gould (20) calculated from these data that in a pool consisting of liver, plasma and red cells, 1.2 grams of cholesterol are turned over each day. It would thus appear that phospholipids are turned over much more rapidly than cholesterol.

B. *Results in patients with the nephrotic syndrome*

In six experiments in five patients with the nephrotic syndrome the mean turnover time of phospholipid phosphorus was 159 hours with a range of 97 to 200 hours. The difference between

TABLE III

CALCULATED VALUES FOR "k" AND TURNOVER TIME (TT)

PATIENT	DIAGNOSIS	"k" Calculated at Time Intervals (hours) 24	48	72	96	$TT = \frac{1}{K}$
P.A.	Normal	1.8×10^{-2}	1.7×10^{-2}	1.5×10^{-2}	1.3×10^{-2}	65
O.B.	Normal	1.6 "	1.8 "	1.9 "	1.4 "	60
A.S.	Normal	1.7 "	1.8 "	1.4 "	1.2 "	65
C.K.	Normal	1.0 "	1.1 "	1.1 "	1.0 "	95
R.C.	Normal	1.3 "	1.5 "	1.3 "	1.1 "	77
F.H.	Normal	1.1 "	1.3 "	1.2 "	1.1 "	85
W.F.1	Nephrosis	0.6 "	1.1 "	1.1 "	1.3 "	97
W.F.2	Nephrosis	0.3 "	0.4 "	0.6 "	0.8 "	189
A.G.	Nephrosis	0.6 "	0.7 "	0.8 "	0.8 "	127
B.D.	Nephrosis	0.5 "	0.6 "	0.7 "	0.7 "	173
J.P.	Nephrosis	0.4 "	0.7 "	0.7 "	0.7 "	159
R.G.	Nephrosis	0.5 "	0.5 "	0.6 "	0.6 "	200
R.Go.	Hypercholesterolemia	0.9 "	1.1 "	1.1 "	0.9 "	100
G.G.	Biliary Cirrhosis	1.2 "	1.4 "	1.4 "	1.6 "	72
H.McC.1	Hypothyroidism untreated	0.5 "	0.5 "	0.6 "	0.6 "	200
H.McC.2	Hypothyroidism treated	0.9 "	1.2 "	1.1 "	1.1 "	93

"k" is the fraction of the phospholipid phosphorus in the "plasma-liver pool" turned over per hour. The value for "Turnover Time" shown in this figure is equal to the reciprocal of the mean of the four values of "k". Turnover Time is expressed in hours.

these results and those in the normals was found to be highly significant statistically (P less than 0.01). This result is consistent with the finding of London, Sabella, and Yamasaki that in a patient with the nephrotic syndrome the half-life time of plasma cholesterol was prolonged (21).

It can be seen in Table III, that in the normals the value of "k" in any one individual is relatively constant. In the patients with nephrosis and in the patient with hypothyroidism, however, the value of "k" calculated at 24 hours is consistently lower than that at later time intervals. This finding indicates that one or more of the assumptions made was not justified in these patients, since if all the assumptions were correct "k" should be constant for each individual. It should be noted, however, that our calculations are based entirely upon the relationship between the simultaneous specific activities of urine phosphate and plasma lipid phosphorus and, even if all the assumptions are discarded, it is still possible to use the calculated value of turnover time as a measure of this relationship. These results thus give statistical confirmation of what was shown graphically in Figure 3, namely, that in relation to the specific activity of urine phosphate, the specific activity of plasma lipid phosphorus increased more slowly in the patients with nephrosis than in the normal individuals.

The slower than normal increase in plasma lipid phosphorus specific activity in these patients may be explained by one of the three following hypotheses:

1. Delay in isotopic equilibrium being reached between urine phosphate and the immediate precursor of liver phospholipids.

2. If we accept the assumptions made for our calculation, a prolonged turnover time of phospholipids in the "plasma-liver pool."

3. Delay in the establishment of isotopic equilibrium between plasma and liver phospholipids.

Hypothesis 1 would indicate abnormal metabolism of the precursors of liver phospholipids. There is at present no evidence for this hypothesis.

According to Hypothesis 2 our data indicate a prolonged turnover time of lipid phosphorus contained in the "plasma-liver pool." Although no data are available on the phospholipid content of the liver in patients with the nephrotic syndrome, Bally and Emerson (22) have shown that in rats with nephrotoxic nephritis the phospholipid content of the liver is normal even in the presence of marked hyperlipemia. If we assume a value of 0.6 gram (twice normal) for the lipid phosphorus content of plasma, a value of 2 grams (normal) (17) for the lipid phosphorus content of the liver, and 159 hours as the turnover time (the average value in our six experiments), it could be calculated that the patients with nephrosis turned over $(2 + 0.6 \text{ gram})/159 \text{ hours} = 0.016$ gram of lipid phosphorus per hour, approximately half the normal amount. However, for reasons discussed below we do not favor this interpretation.

According to Hypothesis 3, the slow increase in plasma phospholipid specific activity is due to delay in isotopic equilibrium being reached between plasma and liver phospholipids. Bearing in mind the observation (9, 10) that virtually all plasma phospholipids are both manufactured and removed from the circulation by the liver, this would mean that the turnover time of plasma phospholipids is prolonged without there necessarily being any abnormality in the turnover time of liver phospholipids.

This hypothesis could explain the variation of the calculated values in the nephrotics of the fraction of lipid phosphorus in the pool turned over per hour (k) as shown in Table III. It will be noted that in each of the nephrotics "k" is lower at 24 hours than at later time intervals. It can be shown by substituting various values for "k" in Equation *4*, that it usually varies in the same direction as the specific activity of lipid phosphorus (L). Since the liver is the main source of plasma phospholipids, it would be expected, and it was indeed found to be the case in the dog (9), that before isotopic equilibrium is reached the specific activity of plasma phospholipids is less than that of liver phospholipids. Delay in the establishment of this equilibrium thus could explain the observed variation in "k."

Hiller, Linder, Lundsgaard, and Van Slyke (23) found a greater than normal increase in plasma lipids in patients with nephrosis after a fat meal and Stanley and Thannhauser (24) found that following oral administration of I^{131} labeled olive oil, radioactivity in plasma lipids reached a higher peak and disappeared more slowly in a patient with nephrosis than in normal individuals. If it is assumed that fat absorption in nephrosis is normal and that the administered fat was completely mixed with plasma lipids, then both of these experiments indicate that in nephrosis a smaller than normal fraction of plasma lipids is removed from the circulation per unit time. These experiments are in agreement with Hypothesis 3, namely, that there is a delay in the attainment of isotopic equilibrium between plasma and liver phospholipids.

As noted earlier our results apply only to the average turnover time of total lipid phosphorus. Zilversmit, Entenman, and Chaikoff (8) found that in the liver of the dog lecithin is turned over more rapidly than cephalin or sphingomyelin. A marked change in the composition of phospholipids alone could thus conceivably account for our findings. Albrink (25) found that in the plasma from two patients with nephrosis the ratio of choline to non-choline containing phospholipids was normal, but she did not determine the ratio of lecithin to sphingomyelin. Further data on the composition of phospholipids in nephrosis as well as turnover studies of separate types of phospholipids would thus be of interest.

C. *Results in other types of hyperlipemia*

In one patient with untreated hypothyroidism the turnover time of phospholipid phosphorus was calculated to be 200 hours, while in the same patient during thyroid therapy it was calculated to be 93 hours. This finding is in agreement with results obtained in experimental animals by Flock, Bollman, and Berkson (12) and data on cholesterol synthesis in hypo- and hyperthyroid rats by Byers, Rosenman, Friedman, and Biggs (26). Since our studies in hypothyroidism are confined to one patient they are not suitable for statistical analysis. However, results in this patient appeared to be similar to those in the patients with the nephrotic syndrome.

The value for turnover time calculated in the patient with familial hypercholesterolemia was intermediate between that in the normals and the patients with nephrosis.

In our one patient with biliary cirrhosis it was calculated that the phospholipid phosphorus turnover time was within normal limits. In this patient the plasma phospholipid concentration was markedly increased. If it is assumed that the phospholipid content of the liver in this patient was not less than normal, it would be concluded that the phospholipid content of the "plasma-liver pool" was larger than normal. It seems likely, therefore, that in this patient a larger than normal quantity of phospholipid phosphorus was turned over per unit time. No conclusions are warranted, of course, from studies in one patient. It is of interest, however, that this finding is in agreement with those of Balfour (*27*) in a group of patients including ten individuals with hyperlipemia due to liver or biliary disease. It is, therefore, suggested that the mechanism of hyperlipemia due to liver disease or biliary obstruction is different from that in the nephrotic syndrome.

SUMMARY

1. In six normal individuals and in eight patients with hyperlipemia, the specific activity of plasma lipid phosphorus and urine phosphate was measured following the intravenous injection of P^{32} labeled inorganic phosphorus.

2. Turnover time of phospholipid phosphorus contained in the plasma plus liver was calculated from these data and in six normal individuals this was estimated to be 60 to 95 hours.

3. In the patients with nephrosis and in a patient with untreated hypothyroidism the specific activity of plasma lipid phosphorus, in relation to that of urine phosphate, increased more slowly than in normal individuals, while in a patient with biliary cirrhosis it increased at a nearly normal rate. Intermediate values were obtained in one patient with familial hypercholesterolemia.

4. It is suggested that the most likely interpretation of the findings in the patients with nephrosis is a delay in the attainment of isotopic equilibrium between plasma and liver phospholipids.

ACKNOWLEDGMENT

The authors wish to express their appreciation to Doctors Isidore Edelman and Arthur K. Solomon for their invaluable advice and criticism. We are also indebted to Doctor Solomon for permission to use the equipment of the Biophysical Laboratory at the Harvard Medical School for the radioactivity determinations.

REFERENCES

1. Zilversmit, D. B., Entenman, C., and Fishler, M. C., On the calculation of "turnover time" and "turnover rate" from experiments involving the use of labeling agents. J. Gen. Physiol., 1943, **26**, 325.
2. Folch, J., Ascoli, I., Lees, M., Meath, J. A., and LeBaron, F. N., Preparation of lipide extracts from brain tissue. J. Biol. Chem., 1951, **191**, 833.
3. Fiske, C. H., and Subbarow, Y., The colorimetric determination of phosphorus. J. Biol. Chem., 1925, **66**, 375.
4. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric Techniques and Tissue Metabolism. Minneapolis, Burgess Publishing Company, 1949, p. 208.
5. Handler, P., and Cohn, D. V., Use of radiophosphorus in studies of glomerular permeability of plasma inorganic phosphate. Am. J. Physiol., 1951, **164**, 646.
6. Govaerts, J., Urinary excretion of phosphate with $^{32}_{15}P$ as indicator. Nature, 1947, **160**, 53.
7. Levenson, S. M., Adams, M. A., Rosen, H., and Laskey-Taylor, F. H., Studies in phosphorus metabolism in man. III. The distribution, exchange and excretion of phosphorus in man using radioactive phosphorus (P^{32}) as a tracer. J. Clin. Invest., 1953, **32**, 497.
8. Zilversmit, D. B., Entenman, C., and Chaikoff, I. L., The measurement of turnover of the various phospholipides in liver and plasma of the dog and its application to the mechanism of action of choline. J. Biol. Chem., 1948, **176**, 193.
9. Fishler, M. C., Entenman, C., Montgomery, L. M., and Chaikoff, I. L., The formation of phospholipid by the hepatectomized dog as measured with radioactive phosphorus. I. The site of formation of plasma phospholipids. J. Biol. Chem., 1943, **150**, 47.
10. Entenman, C., Chaikoff, I. L., and Zilversmit, D. B., Removal of plasma phospholipids as a function of the liver: The effect of exclusion of the liver on the turnover rate of plasma phospholipids as measured with radioactive phosphorus. J. Biol. Chem., 1946, **166**, 15.
11. Sachs, J., Phosphate transport and turnover in the liver. Arch. Biochem., 1951, **30**, 423.
12. Flock, E. V., Bollman, J. L., and Berkson, J., Effect of thyroxine and thiouracil on the rate of phospholipid turnover in the liver of the rat. Am. J. Physiol., 1948, **155**, 402.
13. Popjak, G., and Muir, H., In search of a phospholipin precursor. Biochem. J., 1950, **46**, 103.
14. Tolbert, M. E., and Okey, R., The relative rates of renewal of choline and phosphate in liver phospholipide in the rat. J. Biol. Chem., 1952, **194**, 755.
15. Kennedy, E. P., Synthesis of phosphatides in isolated mitochondria. J. Biol. Chem., 1953, **201**, 399.

16. Kornberg, A., and Pricer, W. E., Jr., Enzymatic esterification of α-glycerophosphate by long chain fatty acids. J. Biol. Chem., 1953, **204**, 345.
17. Man, E. B., Kartin, B. L., Durlacher, S. H., and Peters, J. P., The lipids of serum and liver in patients with hepatic diseases. J. Clin. Invest., 1945, **24**, 623.
18. Weinman, E. O., Chaikoff, I. L., Entenman, C., and Dauben, W. G., Turnover rates of phosphate and fatty acid moieties of plasma phospholipides. J. Biol. Chem., 1950, **187**, 643.
19. London, I. M., and Rittenberg, D., Deuterium studies in normal man: I. The rate of synthesis of serum cholesterol. II. Measurement of total body water and water absorption. J. Biol. Chem., 1950, **184**, 687.
20. Gould, R. G., Lipid metabolism and atherosclerosis. Am. J. Med., 1951, **11**, 209.
21. London, I. M., Sabella, G. F., and Yamasaki, M. M., Studies on the metabolism of cholesterol in normal man and in the nephrotic syndrome. J. Clin. Invest., 1951, **30**, 657.
22. Bally, P., and Emerson, K., Jr., To be published.
23. Hiller, A., Linder, G. C., Lundsgaard, C., and Van Slyke, D. D., Fat metabolism in nephritis. J. Exper. Med., 1924, **39**, 931.
24. Stanley, M. M., and Thannhauser, S. J., The absorption and disposition of orally administered I^{131}-labeled neutral fat in man. J. Lab. & Clin. Med., 1949, **34**, 1634.
25. Albrink, M. J., The choline-containing phospholipids of serum. J. Clin. Invest., 1950, **29**, 46.
26. Byers, S. O., Rosenman, R. H., Friedman, M., and Biggs, M. W., Rate of cholesterol synthesis in hypo- and hyperthyroid rats. J. Exper. Med., 1952, **96**, 513.
27. Balfour, W. M., Human plasma phospholipid formation: A study made with the aid of radiophosphorus. Gastroenterology, 1947, **9**, 686.

THE EFFECTS OF MITRAL VALVULOPLASTY ON CARDIOVASCULAR AND RENAL FUNCTION AT REST AND DURING EXERCISE [1]

By WALTER E. JUDSON, J. D. HATCHER,[2] WILLIAM HOLLANDER,[3] AND MEYER H. HALPERIN

(From the Evans Memorial, Massachusetts Memorial Hospitals, and the Department of Medicine, Boston University School of Medicine, Boston, Mass.)

(Submitted for publication December 27, 1954; accepted April 20, 1955)

The effects of mitral valvuloplasty on general cardiohemodynamic function in patients with mitral stenosis have been reported by many investigators (1–10). Measurements of renal blood flow, glomerular filtration rate, and excretion of sodium and their correlation with changes in cardiac output, blood oxygen measurements, and vascular pressures also have been extensively studied in patients with various types of heart disease, with and without congestive failure, at rest (11, 12) and during exercise (13–15). However, little information is available on the changes in renal circulation or excretion of electrolytes and water in the period early after mitral valvuloplasty (16). The purpose of this paper is to describe these changes in renal function and to correlate them with alterations in cardiovascular responses. Particular effort has been made to determine whether a specific cardiohemodynamic pattern is associated with disturbances of salt and water excretion before and after mitral valvuloplasty, both at rest and during exercise.

METHODS AND MATERIALS

Nine patients who had mitral stenosis and, in some instances, minimal aortic valvular disease, were studied before and after mitral valvuloplasty. A complete clinical description of the cardiovascular status and treatment of each of the patients is indicated in Table I. The laboratory procedures were performed before, and from 2 to 32 weeks after the operation.

The patients were studied in the postabsorptive state without any sedative medication. A double-lumen intracardiac catheter was placed in the right heart and pulmonary artery. An inlying needle was maintained in the brachial artery. Zero point of reference of the pressures was 10 cm. anterior to the back of the patient in the supine position. Pressures were measured by electromanometers [4] and recorded by a direct-writing oscillograph. Mean pressures were determined by electrical integration.

The resistances were calculated from the following formulae: [5]

The "total pulmonary" resistance (actually, total resistance opposing the right ventricle) was obtained from the following equation:

$$R = \frac{PA_m}{CO} \times 1{,}332 \text{ dynes seconds cm.}^{-5}.$$

The "pulmonary arteriolar" resistance (actually, the total pulmonary vascular resistance) was calculated as follows:

$$R = \frac{PA_m - \text{“}PC_m\text{”}}{CO} \times 1{,}332 \text{ dynes seconds cm.}^{-5}.$$

The "total peripheral" resistance (actually, total resistance opposing the left ventricle) was calculated as follows:

$$R = \frac{BA_m \times 1{,}332}{CO} \text{ dynes seconds cm.}^{-5}.$$

[1] This investigation was supported in part by a grant from the National Heart Institute of the National Institutes of Health, U.S.P.H.S.

[2] Present address: Queen's University, Kingston, Ontario, Canada.

[3] United States Public Health Service Fellow in Medicine, Evans Memorial, Massachusetts Memorial Hospitals.

[4] Sanborn Company, Cambridge, Massachusetts.

[5] Vascular resistances were calculated according to formulae devised by Gorlin and his co-workers (17), whose designations are employed here. "Total pulmonary" resistance is defined in terms of the gradient of pressure from the pulmonary arteries to zero, and actually includes the resistance of the pulmonary vessels, that of the mitral valve, as well as the resistance to filling by the left ventricle. Thus, the so-called "total pulmonary" resistance should more correctly be designated as the total resistance opposing the right ventricle. Similarly, calculation of the "pulmonary arteriolar" resistance is based on the pressure gradient between the pulmonary arteries and the "wedge-pressure." If the latter reflects pulmonary venous pressure, then the calculation of "pulmonary arteriolar" resistance actually includes the resistance of the capillaries and venules, and should be called total pulmonary vascular resistance. "Total peripheral" resistance, furthermore, is calculated on the basis of the pressure drop from the large arteries to zero, and actually includes the resistance to filling by the right heart. Therefore, this should more correctly be defined as the total resistance opposing the left ventricle.

The work of the right and left ventricles against pressure was calculated with the following formulae:

$$W_R = \frac{(CI \times 1.055)([PA_m - RA_m] \times 13.6)}{1{,}000}$$

kg. M. per minute per M^2,

$$W_L = \frac{(CI \times 1.055)([BA_m - 5] \times 13.6}{1{,}000}$$

kg. M. per minute per M^2,

PA_m = pulmonary arterial mean pressure in mm. Hg,
"PC_m" = "pulmonary capillary" mean pressure in mm. Hg,
BA_m = brachial arterial mean pressure in mm. Hg,
CO = cardiac output in ml. per second,
1,332 = factor for converting mm. Hg to dynes per $cm.^5$,
W_R = work of right ventricle against pressure,
W_L = work of left ventricle against pressure,
CI = cardiac index, liters per minute per M^2,
RA_m = right atrial pressure in mm. Hg,
1,055 = specific gravity of blood,
5 = assumed left ventricular diastolic pressure in mm. Hg,
13.6 = specific gravity of mercury.

An indwelling catheter was placed in the urinary bladder. After standard priming doses, a sustaining infusion containing inulin (3.3 per cent) and para-amino-hippurate (0.5 per cent) in 3.5 per cent glucose and water was started and given at the rate of 1.2 ml. per minute by means of a calibrated drip.

After a preliminary practice run, at least one and usually two resting cardiac output determinations were made by the direct Fick method. Expired air was collected for two minutes in a Douglas bag while simultaneous blood samples were obtained at a constant rate from the brachial and pulmonary arteries. Immediately before and after the Fick procedure, pressures in the pulmonary artery, right ventricle, and brachial artery were recorded. The resting "pulmonary capillary" pressure was measured after the completion of measurement of the resting cardiac outputs. The volume of inspired air was measured by a Tissot spirometer which supplied outdoor air to the patient. Oxygen and carbon dioxide concentrations in the expired air were determined by the Haldane method (18), duplicate determinations being required to agree within 0.03 vol. per cent. Blood samples were analyzed for oxygen content, capacity and saturation by the method of Van Slyke and Neill (19). Blood oxygen contents were corrected for differences in hematocrit, duplicate determinations being required to agree within 0.10 vol. per cent.

During the control period, three to four urine collections were obtained. Urine was collected at the end of each 10 to 15-minute period by washing out the bladder with 20 ml. of distilled water and evacuating the residual urine by displacement with air. Every twenty minutes arterial blood samples were obtained for the determinations of inulin, para-amino hippuric acid, chloride, sodium, potassium, and hematocrit. Blood and urine concentrations of para-amino hippuric acid were measured by the method of Goldring and Chasis (20) and inulin was determined by the method of Schreiner (21) or Roe, Epstein, and Goldstein (22). Serum and urinary sodium and potassium were determined by the internal standard flame photometer (lithium). Serum and urinary chloride were analyzed by the method of Wilson and Ball (23). Hematocrit was measured in Wintrobe tubes.

Measurements during the effects of exercise were carried out as follows:

With the distal lumen of the catheter wedged in a radicle of the pulmonary artery the patient exercised in the supine position by alternate straight leg-raising from two and one-half to nine and one-half minutes. During the final minute of exercise the cardiac output was measured. At the instant that the exercise was stopped the measurements of brachial artery, "pulmonary capillary," pulmonary arterial, and right ventricular pressures which were being taken were recorded for use in the calculation of the hemodynamic data. (Pressure measurements were also taken and recorded repeatedly during exercise but were not regarded as completely valid because of the marked fluctuations in intrathoracic and intra-abdominal pressures associated with the strenuous exercise of alternate straight leg-raising.) Urine collections were made at the end of the 10-minute period including the time during which the exercise was performed ("the exercise period") and thereafter for three 10 to 15-minute periods. All these procedures were performed after operation in the identical manner as before.

RESULTS

Cardiohemodynamic data for each patient are presented in Table II, and the results in two cases are shown graphically in Figures 1 and 2. The mean data for the group and a statistical analysis are shown in Table III, which is the major basis for the following presentation.

Cardiovascular responses

Cardiac index and oxygen measurements. The mean resting cardiac index for the entire group before the operation was 2.71 liters per minute per square meter of body surface. The mean increase in the resting cardiac index for the group after operation was 0.36 liter per minute per square meter of body surface, which was not statistically significant. The mean cardiac index during exercise was 0.76 L. per min. per M^2 greater after than before operation. In response to exercise all but one patient (C. J.) had rises in cardiac index both before and after operation (average 0.87 L. per min. per M^2 preoperatively and 1.26 L. per min. per M^2 postoperatively). Thus, the response of cardiac index to exercise after operation averaged 0.41 L. per min. per M^2 greater than

TABLE I

Clinical data of the patients before and after mitral valvuloplasty

Patient Age	Sex	Condition	Clinical classification	Symptoms and signs of congestive failure: Hemoptysis	Dyspnea	Veins	Rales	Liver below costal margin *cm.*	Peripheral edema	Rhythm*	Treatment: Digitalis	Diuretic	Low salt diet†	Ventricular hypertrophy by EKG	Arterial hypertension	Valvular lesions (according to clinical and surgical findings)*: M.S.‡	M.I.	A.S.	A.I.	Calcification mitral valve
J. C.		Pre-op.	IV	4+	4+	3+	2+	4	1+	AF	+	+	+	R	0	4+	1+	0	0	4+
43	M	Post-op.	III	0	1+	2+	0	0	0	AF	+	+	+	R	0	2+	1+	0	0	4+
A. L.		Pre-op.	III	0	4+	2+	2+	0	1+	AF	+	+	+	0	0	4+	0	0	0	0
36	F	Post-op.	III	0	1+	2+	0	0	0	AF	+	+	+	0	0	2+	0	0	0	0
C. J.		Pre-op.	IV	0	4+	4+	2+	6	4+	AF	+	+	+	R	+	4+	0	0	0	2+
40	F	Post-op.	III	0	2+	2+	0	2	0	AF	+	+	+	R	0	3+	0	0	0	2+
C. S.		Pre-op.	III	0	4+	2+	1+	0	1+	NSR	+	+	+	R	0	4	0	0	0	1+
37	M	Post-op.	I	0	0	0	0	0	0	NSR	0	0	0	R	0	1+	0	0	0	1+
E. M.		Pre-op.	III	0	4+	0	0§	0	0	NSR	+	+	+	0	+	4+	0	0	0	0
48	F	Post-op.	III	0	2+	0	0	0	0	NSR	+	+	+	0	+	2+	0	0	0	0
C. M.		Pre-op.	III	0	4+	0	0	0	0	NSR	0	0	0	0	0	3+	0	0	0	0
49	M	Post-op.	II	0	1+	0	0	0	0	NSR	0	0	0	0	0	1+	0	0	0	0
E. T.		Pre-op.	III	4+	4+	0	0§	0	0	NSR	+	0	0	0	0	4+	0	0	1+	0
22	F	Post-op.	II	0	1+	0	0	0	0	NSR	+	0	0	0	0	1+	0	0	1+	0
E. C.		Pre-op.	III	2+	4+	2+	0	4	1+	AF	+	0	+	0	0	4+	0	0	1+	0
50	F	Post-op.	II	0	1+	0	0	0	0	AF	+	0	+	0	0	1+	0	0	1+	0
A. C.		Pre-op.	II	0	3+	0	0	0	0	NSR	0	0	0	0	0	4+	0	0	0	0
40	M	Post-op.	I	0	0	0	0	0	0	NSR	0	0	0	0	0	1+	1+	0	0	0
A. S.		Pre-op.	III	4+	3+	0	0	0	0	NSR	+	0	0	0	0	4+	0	0	0	0
39	F	Post-cardiotomy	III	4+	2+	0	0	0	0	NSR	+	0	0	0	0	4+	0	0	0	0

* Key to abbreviations:
AF = Auricular Fibrillation.
NSR = Normal Sinus Rhythm.
R = Right.
M.S. = Mitral Stenosis.
M.I. = Mitral Insufficiency.
A.S. = Aortic Stenosis.
A.I. = Aortic Insufficiency.

† <3 gm. NaCl per day.

‡ Surgical estimation of length of mitral valvular orifice.
1+ = >3.1 cm.
2+ = 2.1–3 cm.
3+ = 1.1–2 cm.
4+ = 0.5–1 cm.

§ Attacks of pulmonary edema occurred in these patients.

TABLE II

Individual cardiohemodynamic data *

Patient Age Sex Body surface area (M^2)	Time relative to operation	Arterial oxygen saturation %		Oxygen consumption *ml./min./M²*		A-V oxygen difference *vol. %*		Cardiac index *L./min./M²*		Heart rate *beats/min.*		Stroke index *ml./beat/M²*	
		R	E	R	E	R	E	R	E	R	E	R	E
J. C. 43 M 1.99	Pre-op.	91	90	163	347	5.23	10.69	3.18	3.23	85	100	37	32
1.96	16 wks. Post-op.	94	95	164	350	5.07	8.48	3.26	4.12	66	98	49	42
A. L. 36 F 1.64	Pre-op.	99	100	148	427	5.86	10.67	2.54	4.00	84	180	30	22
1.63	9 wks. Post-op.	97	99	144	427	5.03	10.48	2.85	4.07	92	140	31	29
C. J. 40 F 1.63	Pre-op.	89	86	180	209	9.04	10.92	1.98	1.91	107	124	18	15
1.57	2 wks. Post-op.	92	95	182	321	6.24	11.46	2.93	2.80	84	98	35	29
C. S. 37 M 1.64	Pre-op.	93	90	179	427	6.73	13.36	2.67	3.19	85	136	31	23
1.71	28 wks. Post-op.	98	98	171	439	4.13	7.45	4.17	5.89	80	110	52	54
E. M. 48 F 1.66	Pre-op.	89	86	127	—	5.66	9.71	2.24	—	77	104	29	—
1.63	20 wks. Post-op.	95	93	131	363	4.80	9.35	2.74	3.88	71	120	39	32
C. M. 49 M 1.69	Pre-op.	100	92	130	367	4.21	7.51	3.09	4.89	63	98	49	50
1.69	32 wks. Post-op.	99	99	125	379	5.04	9.06	2.48	4.19	60	84	41	50
E. T. 22 F 1.49	Pre-op.	94	92	146	377	4.42	8.24	3.28	4.58	62	110	53	42
1.48	17 wks. Post-op.	99	—	144	357	3.75	6.65	3.86	5.36	78	96	50	56
E. C. 50 F 1.43	Pre-op.	95	100	130	280	6.22	11.13	2.14	2.51	48	88	44	28
1.44	2 wks. Post-op.	96	94	149	344	5.12	9.41	2.87	3.65	82	100	35	37
A. C. 40 M 2.10	Pre-op.	96	94	145	470	4.45	9.83	3.25	4.79	78	138	42	35
2.08	6 wks. Post-op.	94	94	124	538	4.97	10.65	2.48	5.06	68	120	36	42
A. S.† 39 F 1.46	Pre-op.	97	99	160	286	4.59	7.84	3.49	3.65	84	112	42	33
1.46	16 wks. Post-op. (Cardiotomy)	96	98	172	425	4.67	7.99	3.67	5.29	79	128	46	41

* R = Rest
E = Exercise
S = Systolic
D = Diastolic
M = Mean

† Not included in mean or statistical summary.
M^2—refers to square meters of body surface area.

TABLE II—*Continued*

Patient Age Sex Body surface area (M^2)	Time relative to operation	Pressures (*mm. Hg*)															
		Pulmonary artery						Mean "pulmonary capillary"		Right ventricular end diastolic		Systemic arterial					
		R			E							R			E		
		S	D	M	S	D	M	R	E	R	E	S	D	M	S	D	M
J. C. 43 M 1.99	Pre-op.	90	45	55	115	55	85	22	32	10	15	120	70	85	145	80	95
1.96	16 wks. Post-op.	58	28	40	80	40	53	20	30	12	15	130	75	90	160	100	120
A. L. 36 F 1.64	Pre-op.	35	20	24	63	40	43	21	30	15	30	140	90	110	178	110	130
1.63	9 wks. Post-op.	33	15	22	48	30	37	14	27	10	12	130	75	90	187	115	140
C. J. 40 F 1.63	Pre-op.	125	70	90	175	80	115	25	35	25	—	190	120	150	200	135	160
1.57	2 wks. Post-op.	78	40	52	110	52	72	19	35	12	26	120	80	100	170	115	125
C. S. 37 M 1.64	Pre-op.	102	47	65	138	61	99	30	41	10	18	130	80	100	170	100	135
1.71	28 wks. Post-op.	50	28	38	80	48	60	22	30	5	10	120	60	90	145	70	100
E. M. 48 F 1.66	Pre-op.	76	38	55	110	52	75	34	55	5	10	170	90	120	265	150	207
1.63	20 wks. Post-op.	47	28	35	88	48	57	28	42	5	12	190	100	130	210	110	145
C. M. 49 M 1.69	Pre-op.	65	30	40	112	52	85	26	55	5	10	135	75	100	180	90	130
1.69	32 wks. Post-op.	32	16	23	60	35	45	15	22	5	5	125	65	90	145	75	110
E. T. 22 F 1.49	Pre-op.	45	20	33	82	46	65	20	40	2	5	105	60	80	125	72	88
1.48	17 wks. Post-op.	42	22	32	58	30	43	18	36	5	5	122	62	79	—	—	—
E. C. 50 F 1.43	Pre-op.	50	30	40	90	55	68	20	38	10	15	105	60	75	—	—	—
1.44	2 wks. Post-op.	53	28	35	86	40	56	18	21	8	10	100	60	75	105	65	80
A. C. 40 M 2.10	Pre-op.	45	35	40	90	60	68	30	48	5	20	138	80	100	140	80	100
2.08	6 wks. Post-op.	33	20	25	58	38	47	15	21	5	10	140	90	110	160	90	120
A. S.† 39 F 1.46	Pre-op.	50	28	35	70	34	50	18	28	5	10	120	70	95	155	90	120
1.46	16 wks. Post-op. (Cardiotomy)	45	23	33	75	38	55	20	30	4	9	130	80	100	—	—	—

TABLE II—*Continued*

Patient Age Sex Body surface area (M^2)	Time relative to operation	Resistances *dynes seconds cm⁻⁵* "Total pulmonary" R	"Total pulmonary" E	"Pulmonary arteriolar" R	"Pulmonary arteriolar" E	Total peripheral R	Total peripheral E	Work of ventricles against pressure *kg. M./min./M²* Right ventricle R	Right ventricle E	Left ventricle R	Left ventricle E
J. C. 43 M 1.99	Pre-op.	700	1,050	420	660	1,080	1,180	2.0	3.3	3.6	4.2
1.96	16 wks. Post-op.	500	520	250	230	1,130	1,190	1.3	2.2	4.0	6.8
A. L. 36 F 1.64	Pre-op.	460	520	60	160	2,110	1,580	0.3	0.8	3.8	7.2
1.63	9 wks. Post-op.	380	450	140	120	1,550	1,690	0.5	1.5	3.5	7.9
C. J. 40 F 1.63	Pre-op.	2,230	2,950	1,610	2,050	3,710	4,100	1.8	—	4.1	4.2
1.57	2 wks. Post-op.	900	1,310	570	670	1,740	2,270	1.7	1.8	4.0	4.8
C. S. 37 M 1.64	Pre-op.	1,190	1,510	640	890	1,830	2,060	2.1	3.7	3.6	6.0
1.71	28 wks. Post-op.	430	480	180	240	1,010	790	2.0	4.2	5.1	8.0
E. M. 48 F 1.66	Pre-op.	1,180	—	450	—	2,580	—	1.6	—	3.7	—
1.63	20 wks. Post-op.	630	720	130	190	2,320	1,830	1.2	2.5	4.9	7.8
C. M. 49 M 1.69	Pre-op.	610	820	210	290	1,530	1,260	1.6	5.3	4.2	8.8
1.69	32 wks. Post-op.	440	510	150	260	1,720	1,240	0.6	2.4	3.0	6.3
E. T. 22 F 1.49	Pre-op.	540	760	210	290	1,310	1,030	1.4	3.9	3.5	5.4
1.48	17 wks. Post-op.	450	430	200	70	1,110	—	1.5	2.9	4.4	—
E. C. 50 F 1.43	Pre-op.	1,050	1,510	520	670	1,960	—	0.9	1.9	2.3	—
1.44	2 wks. Post-op.	680	850	330	530	1,460	1,220	1.1	2.4	2.9	4.2
A. C. 40 M 2.10	Pre-op.	470	540	120	160	1,170	800	1.6	3.3	4.4	6.5
2.08	6 wks. Post-op.	390	360	160	200	1,710	910	0.7	2.7	3.7	8.3
A. S.† 39 F 1.46	Pre-op.	550	750	270	330	1,490	1,800	1.5	2.1	4.5	6.0
1.46	16 wks. Post-op. (Cardiotomy)	490	570	190	260	1,490	—	1.5	3.5	5.0	—

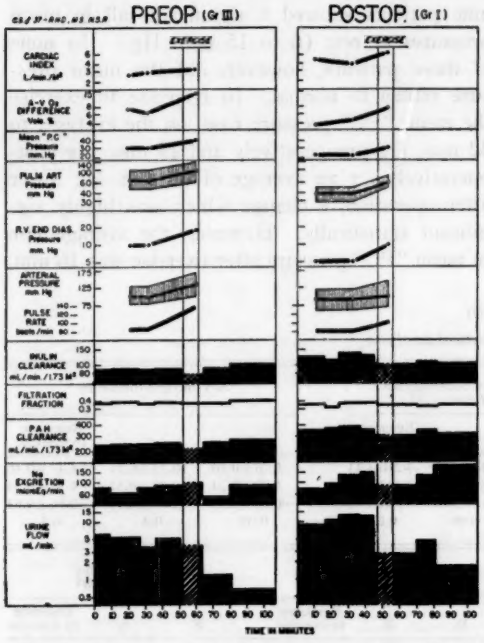


FIG. 1. CHART OF THE CARDIOVASCULAR AND RENAL MEASUREMENTS AT REST AND DURING EXERCISE IN PATIENT C. S. WITH MITRAL STENOSIS BEFORE AND AFTER MITRAL VALVULOPLASTY

Postoperatively this patient showed the greatest improvement in cardio-renal function both at rest and during exercise.

before operation, but this change lacked statistical significance.

The oxygen consumption of each patient at rest was almost identical before and after operation. With exercise the oxygen consumption tended to be higher after than before operation, perhaps reflecting ability to do a greater amount of work. This difference lacked statistical significance in the group as a whole. The arteriovenous oxygen differences both at rest and during exercise were on the average about 1 vol. per cent lower after operation than before. The range of the data was so wide, however, that for this small group statistical analysis did not indicate the change to be significant. The arterial oxygen saturation of seven of the nine patients decreased slightly with exercise preoperatively, but this occurred in only two patients postoperatively. Statistically these changes were not significant. However, in this part of the oxygen dissociation curve small differences in saturation may represent considerable differences in oxygen tension, which we did not measure directly.

Vascular pressures

Pulmonary arterial pressure. All of the patients had abnormally elevated pulmonary arterial pressures at rest both before (average 49 mm. Hg) and after operation (average 34 mm. Hg), this change being physiologically and statistically highly significant ($< P = 0.01$). After exercise, the average mean pulmonary arterial pressure was 78 mm. Hg before operation and 52 mm. Hg after operation. All of the patients had a lower mean pulmonary arterial pressure in response to exercise after than before operation, the

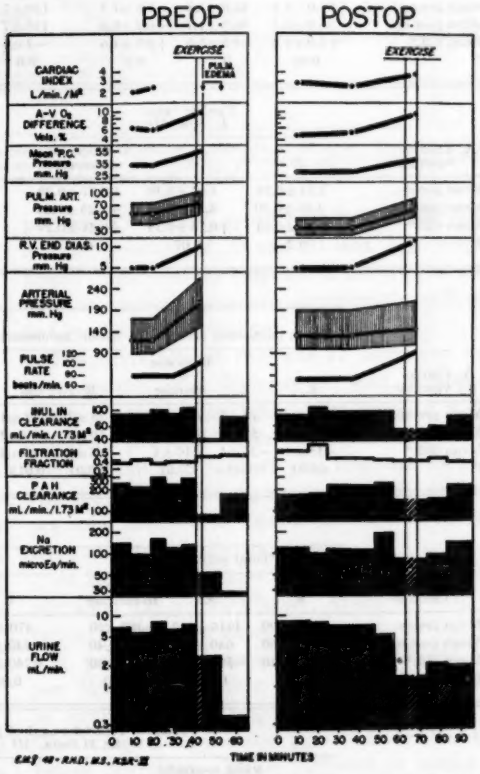


FIG. 2. CHART OF THE CARDIOVASCULAR AND RENAL MEASUREMENTS AT REST AND DURING EXERCISE IN PATIENT E. M. WITH MITRAL STENOSIS BEFORE AND AFTER MITRAL VALVULOPLASTY

Preoperatively the patient developed pulmonary edema during exercise.

decrease being 6 to 43 mm. Hg (average 26 mm. Hg). In response to exercise, the mean pulmonary arterial pressure rise averaged 29 mm. Hg (59 per cent) before operation and 19 mm. Hg (56 per cent) after operation. All these changes had high physiologic and statistical significance.

"Pulmonary capillary" pressure. The average mean "pulmonary capillary" ("PC") pressure at rest before operation was 25 mm. Hg and 19 mm. Hg after operation. Postoperatively six of the nine patients showed a significant fall in mean pressures at rest (6 to 15 mm. Hg). In none of these patients, however, did the mean pressure return to normal. In response to exercise the mean "PC" pressure rose, on the average, to 42 mm. Hg preoperatively and 29 mm. Hg postoperatively, or an average of 13 mm. Hg lower after operation, a change which was highly significant statistically. However, the average rise in mean "PC" pressure after exercise was 16 mm.

TABLE III

Mean data and statistical analysis

A. Cardiovascular	Arterial oxygen saturation % R	E	Response to exercise	Oxygen consumption ml./min./M² R	E	Response to exercise	A-V oxygen difference vol. % R	E	Response to exercise
Mean pre-op.	94.0±1.3	92.0±2.8	−1.8±1.2	150±7	363±30	+210±33	5.76±0.50	10.23±0.57	+4.47±0.46
Mean post-op.	96.0±0.8	96.0±1.7	+0.2±0.6	148±7	391±22	+243±26	4.90±0.24	9.22±0.52	+4.32±0.32
Mean diff.*	+2.0±1.0	+3.6±1.9	+2.0±1.6	−2±4	+31±16	+34±16	−0.85±0.41	−1.01±0.74	−0.16±0.63
P	0.08	0.1	0.2	0.6	0.09	0.07	0.08	0.2	0.8

A. Cardiovascular	Cardiac index L./min./M² R	E	Response to exercise	Heart rate beats/min. R	E	Response to exercise	Stroke index ml./beat/M² R	E	Response to exercise
Mean pre-op.	2.71±0.16	3.64±0.39	+0.87±0.26	77±6	120±9	+43±8	37±4	31±4	−7±2
Mean post-op.	3.07±0.20	4.34±0.31	+1.26±0.25	76±3	107±6	+32±5	41±2	41±4	+0.3±2
Mean diff.*	+0.36±0.24	+0.76±0.35	+0.41±0.19	−1±6	−12±6	−12±7	+4±4	+12±3	+8±3
P	0.2	0.07	0.07	0.9	0.09	0.1	0.3	<0.01	0.033

Pressures (*mm. Hg*)

A. Cardiovascular	Mean pulmonary artery R	E	Response to exercise	Mean "pulmonary capillary" R	E	Response to exercise	Right ventricular end diastolic R	E	Response to exercise	Mean systemic arterial R	E	Response to exercise
Mean pre-op.	49±7	78±7	+29±3	25±2	42±3	+16±2	10±2	15±3	+8±2	102±8	131±15	+25±10
Mean post-op.	34±3	52±4	+19±1	19±1	29±2	+10±1	7±1	12±2	+4±1	95±6	118± 7	+21± 5
Mean diff.*	−15±4	−26±4	−10±3	−6±1	−13±4	− 6±3	−2±2	−6±2	−5±2	−7±6	−14±12	− 5±16
P	<0.01	<0.01	<0.01	<0.01	0.015	0.1	0.2	0.049	0.032	0.3	0.3	0.7

Resistances *dynes seconds cm⁻⁵*

A. Cardiovascular	"Total pulmonary" R	E	Response to exercise	"Pulmonary arteriolar" R	E	Response to exercise	Total peripheral R	E	Response to exercise
Mean pre-op.	940±190	1210±280	+300±80	470±160	650±220	+170±50	1920±280	1720±420	−100±130
Mean post-op.	530± 60	630±100	+ 90±40	230± 50	280± 70	+ 40±30	1530±140	1390±180	−190±150
Mean diff.*	−400±140	−590±180	−210±40	−240±120	−360±170	−130±50	−390±240	−480±350	− 50±170
P	0.021	0.015	<0.01	0.08	0.1	0.042	0.2	0.2	0.7

Work of ventricles against pressure *kg. M./min./M²*

A. Cardiovascular	Right ventricle R	E	Response to exercise	Left ventricle R	E	Response to exercise
Mean pre-op.	1.5±0.2	3.2±0.5	+1.8±0.4	3.7±0.2	6.0±0.7	+2.2±0.6
Mean post-op.	1.2±0.1	2.5±0.3	+1.3±0.2	3.9±0.3	6.8±0.5	+2.9±0.4
Mean diff.*	−0.3±0.2	−0.6±0.5	−0.2±0.1	+0.2±0.3	+0.9±0.7	+0.9±0.6
P	0.1	0.2	0.1	0.5	0.3	0.1

TABLE III—*Continued*

B. Renal	Clearance *ml./min./1.73 M²* PAH Rest	PAH Exercise	PAH Response to exercise	PAH Recovery	Inulin Rest	Inulin Exercise	Inulin Response to exercise	Inulin Recovery
Mean pre-op.	318±31	195±31	−138±22	294±35	100± 7	64±10	−38±12	102± 8
Mean post-op.	370±47	283±51	− 87±11	351±42	117±10	90±14	−26± 6	129±12
Mean diff.	+42±38	+82±47	− 56±26	+48±43	+16± 9	+24±22	+12±16	+14±11
P	0.3	0.1	0.08	0.3	0.1	0.3	0.5	0.3

B. Renal	F.F. % Rest	F.F. % Exercise	F.F. % Response to exercise	F.F. % Recovery	UV *ml./min.* Rest	UV *ml./min.* Exercise	UV *ml./min.* Response to exercise	UV *ml./min.* Recovery	$U_{Na}V$ *MicroEq./min.* Rest	$U_{Na}V$ *MicroEq./min.* Exercise	$U_{Na}V$ *MicroEq./min.* Response to exercise	$U_{Na}V$ *MicroEq./min.* Recovery
Mean pre-op.	33±2	34±5	+2±3	36±3	4.8±1.1	1.7±0.5	−4.0±0.9	2.5±1.3	107±17	62±11	−45±17	101±39
Mean post-op.	34±1	34±2	+0.3±2	34±3	6.7±1.4	2.9±0.6	−4.0±1.3	3.6±0.9	125±23	86±18	−40±11	121±20
Mean diff.	+1±2	0±3	−2±4	0±3	—	—	0.2±1.8	—	—	—	− 4±14	—
P	0.5	—	0.6	—	—	—	>0.9	—	—	—	0.8	—

B. Renal	U_KV *MicroEq./min.* Rest	U_KV *MicroEq./min.* Exercise	U_KV *MicroEq./min.* Response to exercise	U_KV *MicroEq./min.* Recovery	$U_{Cl}V$ *MicroEq./min.* Rest	$U_{Cl}V$ *MicroEq./min.* Exercise	$U_{Cl}V$ *MicroEq./min.* Response to exercise	$U_{Cl}V$ *MicroEq./min.* Recovery
Mean pre-op.	52± 7	34±4	−19±7	52±7	80±16	60±10	− 32±16	82±21
Mean post-op.	68±11	43±8	−24±8	63±7	126±20	92±16	− 34±10	125±18
Mean diff.	—	—	− 7±8	—	—	—	−0.2±13	—
P	—	—	0.4	—	—	—	>0.9	—

* In occasional cases, measurements of some variables were available preoperatively but not postoperatively, or *vice versa*. These are included in the mean pre- and postoperative data. Occasional discrepancies between the means, the individual differences, and the difference between average pre- and postoperative values for the group are due to inclusion of these occasional unpaired data.

Hg (64 per cent) before, and 10 mm. Hg (53 per cent) after operation, a change which was not statistically significant. However, in three patients the rise in "PC" pressure following exercise was considerably lower (12 to 22 mm. Hg) after operation.

Right ventricular end-diastolic pressure. The average right ventricular end-diastolic (R.V.D.) pressure at rest before operation was 10 mm. Hg and after operation 7 mm. Hg. After exercise the average R.V.D. pressure was 15 mm. Hg preoperatively and 12 mm. Hg postoperatively. In response to exercise the R.V.D. pressure increased on the average 8 mm. Hg before operation and only 4 mm. Hg after operation, a change which was just statistically significant.

Arterial pressure, heart rate, stroke index

The average mean arterial pressure at rest, 102 mm. Hg preoperatively and 95 mm. Hg postoperatively, showed no significant change. However, postoperatively two patients, A. L. and C. J. (the latter patient having associated arterial hypertension) showed appreciable falls in the mean arterial pressure of 20 and 50 mm. Hg, respectively. Likewise, the response of the arterial pressure to exercise was not significantly different before and after operation.

The heart rate at rest was 77 beats per min. preoperatively and 76 beats per min. postoperatively for the group, while after exercise it was 120 beats per min. before and 107 beats per min. after operation. Similarly, in response to exercise the heart rate was, on the average, 12 beats per min. lower after operation. These changes were not statistically significant.

At rest the slight rise in the stroke index after operation was not significant. However, with exercise the average stroke index was 31 ml. per min. per M^2 preoperatively and 41 ml. per min. per M^2 postoperatively. Likewise, the increase in stroke index in response to exercise was on the average 8 ml. per min. per M^2 more after than before operation. These increases in stroke index were significant and reflected the associated increases in cardiac index and reductions in heart rate after operation.

Vascular resistances (in units of dynes seconds cm.$^{-5}$)

The *"total pulmonary" resistance,* which includes the calculated resistance of the mitral valve itself, is normally under 200 units at rest. Postoperatively there was a considerable and statistically significant decrease in this resistance as calculated from the data obtained during rest, the average declining from 940 to 530 units. The resistances thus remained elevated above normal even after operation. The resistances calculated from the measurements obtained following exercise likewise dropped (on the average from 1,210 to 630 units) after operation, which is also highly significant statistically. While some patients had only negligible changes in resistance as a result of operation, the direction of the change was uniformly downward.

In response to exercise, "total pulmonary" resistance in normal subjects usually decreases. In eight patients in whom data were obtained both at rest and after exercise pre- and postoperatively, the resistance rose considerably following exercise. The average rise was, however, much less after operation (90 units) than before (300 units). This change was highly significant statistically.

"Pulmonary arteriolar" resistance at rest is normally up to 100 units, and it usually decreases with exercise. In this group, the highly elevated preoperative resting value (average 470 units) declined after operation to 230. Following exercise the "pulmonary arteriolar" resistance was increased (average 650 units) preoperatively, and declined to 280 units after operation. Although most of the patients had lower "pulmonary arteriolar" resistances both at rest and following exercise after operation, the average decreases for the entire group lacked statistical significance. However, the average increase in "pulmonary arteriolar" resistance in response to exercise was 170 units before operation and was only 40 units after operation, a reduction which was statistically significant.

The above calculated resistances indicate that about half the decrease in resistance opposing the right ventricle, both at rest and after exercise, can be accounted for in terms of a reduction in the resistance of the pulmonary vessels, and the other half in terms of a lessening of the resistance beyond the lungs, namely of the mitral valve itself.

The "total peripheral" resistance after operation tended to be less at rest and following exercise than before operation, but these changes were small and not statistically significant.

Ventricular work against pressure (in units of kg. M. per min. per M^2)

The *work of the right ventricle* against pressure (normally less than 1.0 unit) was abnormally high at rest preoperatively (1.5 units) and declined postoperatively to 1.2 units. With exercise, the average right ventricular work rose to 3.2 units preoperatively and to only 2.5 units postoperatively. Thus, the considerable decrease in pulmonary arterial pressure with operation tends to reduce the amount of work by the right ventricle to a greater degree than the slight rise in cardiac output that acts to increase it. As a result of the balances of these two factors, the net effect is a reduction in the right ventricular work, but one which is not statistically significant.

The *work of the left ventricle* against pressure at rest tended to rise slightly after operation because the increase in cardiac output was greater than the fall in peripheral arterial pressure. However, neither the average values of the left ventricular work at rest nor the responses to exercise after operation were significantly greater than before operation.

Renal responses before and after mitral valvuloplasty

Individual renal data are presented in Table IV, and group means and their statistical analysis in Table III.

At rest. Preoperatively all the patients had an abnormally low renal plasma flow (average 318 ml. per min. per 1.73 M^2) while five patients (C. S., E. M., C. M., E. C., A. C.) also had lower than normal glomerular filtration rates (mean value for the entire group was 100 ml. per min. per 1.73 M^2). Although after operation the renal plasma flow and glomerular filtration rate at rest showed significant increases to or towards normal in three patients, C. S., E. T., E. C., the postoperative renal plasma flow (mean 370 ml. per min. per 1.73 M^2) and glomerular filtration rate (mean 117 ml. per min. per 1.73 M^2) for the group as a whole did not show a statistically significant increase. Since intake of dietary salt was not regu-

lated, the influence of the operation upon the excretion of electrolytes at rest could not be accurately evaluated.

During exercise. During the exercise period the mean renal plasma flow was 195 ml. per min. per 1.73 M^2 preoperatively and 283 ml. per min. per 1.73 M^2 postoperatively. Before operation the renal plasma flow decreased in response to exercise, on the average, 138 ml. per min. per 1.73 M^2 or 43 per cent, and the glomerular filtration rate fell, on the average, 38 ml. per min. per 1.73 M^2, or 38 per cent. After operation, exercise produced only about half as much decrease in the renal plasma flow (mean reduction 87 ml. per min. per 1.73 M^2, or 23 per cent), but this alteration just lacked statistical significance for the group. Likewise, the glomerular filtration rate was lowered only half as much after operation (mean reduction 26 ml. per min. per 1.73 M^2, or 22 per cent), but again the change lacked statistical significance for the group.

Preoperatively, associated with the reduction caused by exercise in renal plasma flow and glomerular filtration rate, there were decreases in urine flow and the excretion of sodium, and usually of chloride, except in one patient, C. S. Likewise, after operation in all but one patient, J. C., there was a decrease in sodium excretion during the exercise period. In general, except for patient E. T., the reduction in sodium excretion during the exercise period paralleled the fall in the renal plasma flow and glomerular filtration rate. However, in response to exercise the decrease in sodium excretion was not significantly different be-

TABLE IV

Individual renal data

Patient Age Sex Body surface area (M^2)	Time relative to operation	Procedure	Periods	C_{PAH} *ml./min./ 1.73 M^2*	C_{IN} *ml./min./ 1.73 M^2*	F.F. %	UV *ml./min.*	$U_{Na}V$ *MicroEq./ min.*	$U_{Cl}V$ *MicroEq./ min.*	$U_{K}V$ *MicroEq./ min.*
J. C.	16 wks.	Control	1–5	451	124	28	11.0	132	126	52
43 M	Post-op.	Exercise 6 min.	6	336	100	30	5.5	142	138	39
1.96		Recovery	7–9	384	113	29	6.0	166	180	56
A. L.	Pre-op.	Control	1–5	330	114	35	10.0	121	87	35
36 F		Exercise 7 min.	6	229	97	42	2.7	67	75	35
1.64		Recovery	7–9	306	115	38	10.0	282	170	64
1.63	9 wks.	Control	1–4	305	121	40	12.0	131	128	45
	Post-op.	Exercise 9½ min.	5	189	84	44	5.2	61	78	32
		Recovery	6–7	318	139	44	9.5	130	135	45
C. J.*	Pre-op.	Control	1–3	347	—	—	2.5	96	95	75
40 F		Exercise 5 min.	4	124	—	—	0.15	33	40	24
1.63		Recovery	5–6	283	—	—	0.91	63	76	60
1.57	2 wks.	Control	1–3	378	—	—	3.2	56	83	111
	Post-op.	Exercise 5 min.	4	290	—	—	1.3	24	28	34
		Recovery	5–7	394	—	—	1.3	63	74	70
C. S.	Pre-op.	Control	1–4	256	95	37	5.0	80	73	39
37 M		Exercise 8 min.	5	220	82	37	3.9	102	101	44
1.64		Recovery	6–7	267	104	39	0.94	74	59	41
1.71	28 wks.	Control	1–4	381	131	34	11.0	116	98	88
	Post-op.	Exercise 9½ min.	5	318	106	33	2.8	89	97	63
		Recovery	6–7	339	110	32	3.2	170	152	80
E. M.	Pre-op.	Control	1–5	267	98	37	7.0	127	79	35
48 F		Exercise 2½ min.	6	94	42	44	2.7	54	36	17
1.66		Recovery	7	181	83	46	0.40	29	33	27
1.63	20 wks.	Control	1–4	215	101	47	7.3	132	119	37
	Post-op.	Exercise 6 min.	5	146	55	37	1.4	84	84	22
		Recovery	6–7	199	87	38	2.1	119	114	49

TABLE IV—*Continued*

Patient Age Sex Body surface area (M^2)	Time relative to operation	Procedure	Periods	C_{PAH} *ml./min./1.73 M^2*	C_{IN} *ml./min./1.73 M^2*	F.F. %	UV *ml./min.*	$U_{Na}V$ *MicroEq./min.*	$U_{Cl}V$ *MicroEq./min.*	$U_{K}V$ *MicroEq./min.*
C. M.	Pre-op.	Control	1–5	381	106	28	5.8	170	150	79
49 M		Exercise 8 min.	6	236	75	32	1.1	83	66	44
1.69		Recovery	7–8	430	124	29	3.9	130	114	77
1.69	32 wks.	Control	1–3	303	91	30	1.9	135	158	77
	Post-op.	Exercise 7 min.	4–5	255	84	32	2.4	94	123	67
		Recovery	6	291	100	34	3.5	107	137	88
E. T.	Pre-op.	Control	1–3	486	127	26	4.5	47	64	51
22 F		Exercise 6½ min.	4	324	36	11	0.43	34	41	27
1.49		Recovery	5–6	392	115	29	0.41	28	42	37
1.48	17 wks.	Control	1–3	689	169	25	1.0	85	67	16
	Post-op.	Exercise 5 min.	4	634	165	26	0.48	70	54	16
		Recovery	5–6	625	173	28	0.34	81	64	22
E. C.	Pre-op.	Control	1–3	206	82	40	0.28	6	12	35
50 F		Exercise	—	—	—	—	—	—	—	—
1.43		Recovery	—	—	—	—	—	—	—	—
1.44	2 wks.	Control	1–4	368	127	35	6.3	286	266	106
	Post-op.	Exercise 7 min.	5	269	94	35	4.9	192	181	84
		Recovery	6–8	385	125	33	2.5	226	216	77
A. C.	Pre-op.	Control*	1–3	268	76	28	3.4	780	778	66
40 M		Exercise 7 min.	4	140	54	39	0.80	204	216	44
2.10		Recovery	5–7	197	69	35	1.0	221	242	56
2.08	6 wks.	Control	1–4	242	72	29	6.6	51	85	79
	Post-op.	Exercise 8 min.	5	108	36	33	2.5	14	41	34
		Recovery	6–8	225	84	37	4.4	27	55	76
A. S.	Pre-op.	Control	1–3	589	121	20	4.7	148	120	35
39 F		Exercise	—	—	—	—	—	—	—	—
1.46		Recovery	—	—	—	—	—	—	—	—
1.46	16 wks.	Control	1–3	513	133	26	11.0	148	105	31
	Post-op. (Cardiotomy)	Exercise 6 min.	4	265	78	29	0.08	21	17	11
		Recovery	5	355	110	31	0.20	7	8	15

* Patient was given 300 ml. 5 per cent NaCl I.V.

fore and after operation. The significant decrease in the excretion of potassium during exercise both before and after operation tended to parallel the changes in the glomerular filtration rate. The magnitude of the decrease in the excretion of potassium in response to exercise was not significantly different preoperatively and postoperatively.

Recovery from exercise. Persistently lower values of glomerular filtration rate and renal plasma flow lasting from 13 to 45 minutes after exercise were observed in four patients (A. C., C. J., E. M., and E. T.) before operation. Two of these patients (E. M. and E. T.) developed clinical pulmonary edema during exercise. Three other patients (A. L., C. S., C. M.), none of whom developed pulmonary edema, had a prompt restoration in the renal hemodynamic measurements after exercise and a return to control values within 22 to 45 minutes. Postoperatively, the renal plasma flow tended to return more rapidly and completely to the pre-exercise values during the same period of recovery.

Before operation all except two patients (A. L., C. S.) had reductions in sodium, chloride and usually water excretion during exercise which *persisted* during the recovery period. In the recovery phase sodium and water retention in most of the patients appeared to be independent of the changes in the glomerular filtration rate, since the latter measurements frequently had already returned to the control values. After operation all patients recovered more readily from the exercise-

induced reductions of sodium excretion except for three patients (E. C., C. M., A. C.) who had a persistent decrease in sodium excretion after exercise.

DISCUSSION

There was no consistent relationship between the cardiohemodynamic and the renal functional abnormalities observed in these patients before or after operation. No correlation could be found to exist between the absolute levels of the cardiac output and the renal plasma flow and glomerular filtration rate at rest or during exercise. After mitral valvuloplasty three patients (C. S., E. T., E. C.) showed significant increases in renal plasma flow at rest, and four (C. J., E. M., C. M., E. T.) had smaller decreases in renal plasma flow during exercise. Of the two patients (C. S., E. T.) who had appreciable increases in the glomerular filtration rate at rest, one (E. T.) had a smaller decrease in the glomerular filtration rate in response to exercise after operation. However, these changes in renal plasma flow and glomerular filtration rate were not invariably related to improvement in cardiac output. For example, one patient in the group (C. S.) who had the greatest increase in cardiac output in response to exercise postoperatively had a fall in the renal hemodynamic measurements equal to that found preoperatively. On the other hand, the cardiac output response to exercise was similar before and after operation in patient C. J. whereas the renal plasma flow during the exercise period showed a lesser fall after than before operation.

Prior to operation two patients (E. M., E. T.) developed cardiac asthma during exercise with markedly elevated "pulmonary capillary" pressures, and also had marked reductions in glomerular filtration rate and renal plasma flow and persistent decreases in the excretion of salt and water. After operation they had no cardiac asthma with exercise and smaller decreases in renal plasma flow, glomerular filtration rate, or excretion of salt and water. However, the decreases in renal plasma flow and glomerular filtration rate during the exercise period in the group as a whole could not be correlated with any abnormal increases in mixed arteriovenous oxygen difference or levels of central vascular pressures before or after operation.

Preoperatively the return of the renal hemodynamic measurements to or towards control levels after the exercise period was not consistently related to the prior increase in cardiac output during exercise. However, in some patients after operation there appeared to be an association between improvement in the cardiac output responses to exercise and speed of return of the renal hemodynamic measurements to control levels after exercise. In several patients before operation the decreases in the excretion of sodium and water persisted for some time after exercise. In some cases they continued after the glomerular filtration rates had returned to control values. These decreases were therefore attributed, at least in part, to increased renal tubular reabsorption of salt and water. After operation, however, these decreases in renal function in general persisted for shorter periods. In only one patient (A. C.) in whom an increased renal tubular reabsorption of sodium persisted after exercise did a marked increase in the hematocrit (suggesting a decrease in blood volume with hemoconcentration) occur during exercise both before and after mitral valvuloplasty. It is possible that these two factors may have directly influenced the renal tubular reabsorption of sodium. In other patients during exercise, smaller increases in hematocrit occurred and appeared to contribute little, if at all, to the retention of sodium.

None of the patients after operation had a return to normal of their cardiohemodynamic or their renal functions in response to exercise. Several patients, on the other hand, had a return of individual functions to or almost to normal values at rest. In some, the reductions in urine flow, sodium excretion, renal plasma flow, and glomerular filtration rate were less marked especially in response to exercise than before operation. Likewise in some, the cardiohemodynamic abnormalities were less marked both at rest and in response to exercise than before operation. However, there was no consistent parallelism between these two types of improvement, particularly if measurements of only a single renal or circulatory function was considered. However, there was a fairly consistent trend for those patients with the more normal cardiohemodynamic responses to exercise to show the more normal renal responses.

In general, symptomatic improvement paralleled

improvement in the cardiohemodynamic, or the renal function, or both. However, in some patients marked subjective improvement was noted with little change in either the cardiovascular or renal measurements. In fact, one patient (A. S.) in whom the left auricle was opened but no fracture of the mitral valve accomplished because of technical difficulties had marked symptomatic improvement for about six months without any significant alterations in cardiovascular or renal function.

Some reports on physiologic data obtained from patients with mitral stenosis following mitral valvuloplasty indicate that maximum improvement in the hemodynamic measurements may not appear immediately (24), or early, but usually several months after operation (25–27). The hemodynamic observations in our patients, who were studied early after operation (2 to 32 weeks), were believed at the time to represent maximum states of improvement. Cardiac catheterizations have been repeated in a majority of them one and one-half to two years postoperatively, and the hemodynamic responses in some cases found to be more normal and in others more abnormal than the initial postoperative studies reported here, and a few, actually more abnormal than the preoperative values (28).

SUMMARY

Cardiovascular and renal measurements were made at rest and following exercise in nine patients with mitral stenosis before and within 2 to 32 weeks after mitral valvuloplasty. Cardiohemodynamic measurements after operation showed a slight, but not statistically significant, increase in the cardiac index, and a significant increase in the stroke index, since the heart rates tended to be slower. The pulmonary arterial and "pulmonary capillary" pressures were significantly lower. The right ventricular end-diastolic pressure was not significantly reduced at rest, but after exercise it was significantly less than before operation. The calculated "total pulmonary" resistance (which includes the resistance imposed by the mitral valve) was significantly reduced, but the "pulmonary arteriolar" resistance was not. The increase in the "pulmonary arteriolar" resistance in response to exercise was not so great after operation. The resistance opposing the right ventricle was decreased after operation apparently because of approximately equal reductions in the resistance of the pulmonary vessels and that imposed by the stenosis of the mitral valve. There was a slight reduction in the work of the right ventricle and a slight increase in the work of the left ventricle after operation but neither was statistically significant.

Preoperative resting renal plasma flow was approximately half and glomerular filtration rate about four-fifths of normal values. After operation the resting renal plasma flow and glomerular filtration rate were both increased, on the average, by about one-sixth, the latter approaching normal. In response to exercise the renal plasma flow fell, on the average, 43 per cent before operation and only one-half that much after operation, a significant improvement. The glomerular filtration rate decreased in response to exercise, on the average, 38 per cent before operation and only about two-thirds that much after operation. This improvement was not statistically significant. The considerable decreases in urine flow, excretion of sodium, chloride and potassium in response to exercise, which tended to parallel the changes in the glomerular filtration rate, were not significantly different in magnitude after operation as compared with before. Although all the renal functions tended to recover from exercise more rapidly after operation, the mean data showed no significant differences from the preoperative responses.

In this group of patients mitral valvuloplasty produced varying degrees of improvement in the cardiovascular and renal functions both at rest and after exercise. In none of the patients, however, did the cardio-renal hemodynamic measurements return to normal values. A general improvement in cardiovascular function in individual patients was sometimes associated with increases in renal plasma flow, glomerular filtration rate and the ability to excrete salt and water during and after exercise. However, postoperative increases in salt and water excretion could not be consistently correlated with any specific change in cardiovascular or renal function, or both.

ACKNOWLEDGMENT

The authors wish to thank Dr. Robert W. Wilkins for his excellent suggestions and criticisms.

REFERENCES

1. Dexter, L., Gorlin, R., Lewis, B. M., Haynes, F. W., and Harken, D. E., Physiologic evaluation of patients with mitral stenosis before and after mitral valvuloplasty. Tr. Am. Climat. & Clin. A., 1950, **62**, 170.
2. Draper, A., Heimbecker, R., Daley, R., Carrol, D., Mudd, G., Wells, R., Falholt, W., Andrus, E. C., and Bing, R. J., Physiologic studies in mitral valvular disease. Circulation, 1951, **3**, 531.
3. Lukas, D. S., and Dotter, C. T., Modifications of the pulmonary circulation in mitral stenosis. Am. J. Med., 1952, **12**, 639.
4. Jordan, P., and Hellems, H. K., Mitral valve surgery. A critical analysis. Surg., Gynec. & Obst., 1952, **95**, 689.
5. Janton, O. H., Glover, R. P., O'Neill, T. J. E., Gregory, J. E., and Froio, G. F., Results of the surgical treatment for mitral stenosis. Analysis of one hundred consecutive cases. Circulation, 1952, **6**, 321.
6. Hurwitt, E. S., Bloomberg, A., Aaron, R., Jezer, A., and Young, D., An appraisal of the surgical treatment of mitral stenosis. Ann. Surg., 1953, **138**, 219.
7. Cosby, R. S., Griffith, G. C., Levinson, D. C., Zinn, W. J., Oblath, R. W., Dimitroff, S. P., and Herman, L. M., The physiologic evaluation of patients before and after mitral commissurotomy. Dis. of Chest, 1953, **23**, 499.
8. Baranofsky, I. D., Haddy, F. J., Ferrin, A. L., and Borden, C. W., Cardiopulmonary dynamics of mitral stenosis. A clinical and experimental study. Surgery, 1953, **34**, 347.
9. Curti, P. C., Cohen, G., Castleman, B., Scannell, J. G., Friedlich, A. L., and Meyers, G. S., Respiratory and circulatory studies in patients with mitral stenosis. Circulation, 1953, **8**, 893.
10. Werkö, L., Biörck, G., Crafoord, C., Wulff, H., Krook, H., and Eliasch, H., Pulmonary circulatory dynamics in mitral stenosis before and after commissurotomy. Am. Heart J., 1953, **45**, 477.
11. Merrill, A. J., Edema and decreased renal blood flow in patients with chronic congestive heart failure: evidence of "forward failure" as the primary cause of edema. J. Clin. Invest., 1946, **25**, 389.
12. Eichna, L. W., Farber, S. J., Berger, A. R., Earle, D. P., Rader, B., Pellegrino, E., Albert, R. E., Alexander, J. D., Taube, H., and Youngwirth, S., Cardiovascular dynamics, blood volumes, renal functions and electrolyte excretions in the same patients during congestive heart failure and after recovery of cardiac decompensation. Circulation, 1953, **7**, 674.
13. Merrill, A. J., and Cargill, W. H., The effect of exercise on the renal plasma flow and filtration rate of normal and cardiac subjects. J. Clin. Invest., 1948, **27**, 272.
14. Werkö, L., Varnauskas, E., Eliasch, H., Ek, J., Bucht, H., Thomasson, B., and Bergström, J., Studies on the renal circulation and renal function in mitral valvuluar disease. I. Effect of exercise. Circulation, 1954, **9**, 687.
15. Judson, W. E., Hollander, W., Friedman, I. H., Hatcher, J. D., and Halperin, M. H., Studies on congestive failure: The effects of exercise, salt loading and of intravenous Apresoline on cardiorenal hemodynamics and the excretion of sodium and water. J. Clin. Invest., 1953, **32**, 579.
16. Judson, W. E., Hatcher, J. D., Hollander, W., Halperin, M. H., and Friedman, I. H., Cardiorenal hemodynamics and excretion of sodium and water, at rest and during exercise, in patients with mitral stenosis before and after mitral valvuloplasty. Clin. Research Proc., 1953, **1**, 75.
17. Gorlin, R., Haynes, F. W., Goodale, W. T., Sawyer, C. G., Dow, J. W., and Dexter, L., Studies of the circulatory dynamics in mitral stenosis. II. Altered dynamics at rest. Am. Heart J., 1951, **41**, 30.
18. Haldane, J. S., Methods of Air Analysis. London, Griffin, 1912.
19. Van Slyke, D. D., and Neill, J. M., The determination of gases in blood and other solutions by vacuum extraction and manometric measurement. I. J. Biol. Chem., 1924, **61**, 523.
20. Goldring, W., and Chasis, H., Hypertension and Hypertensive Disease. New York, The Commonwealth Fund, 1944.
21. Schreiner, G. E., Determination of inulin by means of resorcinol. Proc. Soc. Exper. Biol. & Med., 1950, **74**, 117.
22. Roe, J. H., Epstein, J. H., and Goldstein, N. P., A photometric method for the determination of inulin in plasma and urine. J. Biol. Chem., 1949, **178**, 839.
23. Wilson, D. W., and Ball, E. G., A study of the estimation of chloride in blood and serum. J. Biol. Chem., 1928, **79**, 221.
24. Lev, R., Connolly, D. C., Kirklin, J. W., and Wood, E. H., The immediate hemodynamic effects of mitral commissurotomy during surgery. Surg. Forum, 1953, **4**, 13.
25. Baker, C., Brock, R. C., Campbell, M., and Wood, P., Valvotomy for mitral stenosis. A further report, on 100 cases. Brit. M. J., 1952, **1**, 1043.
26. Ellis, F. H., Kirklin, J. W., Parker, R. L., Burchell, H. B., and Wood, E. H., Mitral commissurotomy. An over-all appraisal of clinical and hemodynamic results. Arch. Int. Med., 1954, **94**, 774.
27. Sancetta, S. M., Mueller, R. P., and Gillespie, D. G., Preoperative, six-week, and twelve-month postoperative hemodynamic studies in patients undergoing mitral commissurotomy. J. Lab. & Clin. Med., 1953, **42**, 945.
28. Judson, W. E., Physiological evaluation of postoperative results of surgical treatment of mitral stenosis. Presented March 22, 1954 at a Symposium on Current Problems in Mitral Valvular Disease at the Massachusetts Memorial Hospitals, Boston, Mass., To be published.

CONDITIONS OF FORMATION OF EUGLOBULIN-LIKE PRECIPITATES FROM SERUM PROTEINS AND CHONDROITIN SULFATE[1]

By JACQUES BADIN[2] AND MAXWELL SCHUBERT

(From the Departments of Chemistry and Medicine, and the Study Group on Rheumatic Diseases, New York University College of Medicine, New York, N. Y.)

(Submitted for publication March 10, 1955; accepted March 30, 1955)

The present work constitutes a study of the conditions under which chondroitin sulfate and serum proteins can produce insoluble complexes which behave like euglobulins. Euglobulins are characterized by insolubility in distilled water, solubility in dilute salt solutions and reprecipitability by dialysis of such salt solutions against distilled water.

When potassium chondroitin sulfate is added to normal human serum, no apparent change occurs, but on dialysis a larger amount of euglobulin precipitates. Table I shows an example. To 6 ml. samples of human serum, diluted with an equal volume of water, were added different amounts of potassium chondroitin sulfate (1). Samples were dialyzed 48 hours against a citrate-phosphate buffer, M/150, pH 5.5, according to the method of Sandor, Sabetay, and Vargues (2). After dialysis, euglobulins were centrifuged down, washed with buffer and the amount of proteins precipitated determined using the biuret reaction (3).

As expected from the foregoing data, potassium chondroitin sulfate added to serum that has been previously dialyzed and centrifuged causes a further precipitate which appears as extra euglobulin. The effect of variation in salt concentration, in concentration of chondroitin sulfate and protein, and in pH, in this reaction have been studied.

METHODS

The protein solution used to study precipitation with chondroitin sulfate was human serum freed of euglobulin by dilution with one volume of water and dialysis for 3 to 4 days against a citrate-phosphate buffer, .01 M, pH 4.5 to 5.5. Euglobulins were removed by centrifugation and the supernatant was diluted with buffer until the concentration of total proteins, as determined with the biuret reaction of Jayle, Boussier, and Badin (3), was 10 mg. per ml. For some purposes (as experiment c) a similar euglobulin-free diluted serum was made using distilled water throughout instead of buffer. These preparations are called "diluted dialyzed serum" in buffer and in water. Protein precipitated from these preparations by chondroitin sulfate under various conditions was determined by the biuret reaction on the water washed precipitates in the following experiments.

RESULTS

(a) To a series of tubes, each containing 5 ml. of the diluted dialyzed serum in buffer pH 5.1, were added increasing amounts of NaCl and the volumes were made to 10 ml. with water. The concentration of salt varied from 0.01 M to 0.3 M. Potassium chondroitin sulfate (2 mg. in 1 ml.) was added to each tube and the precipitates were centrifuged after standing 2 to 4 hours at room temperature. Figure 1 shows how increasing the concentration of NaCl decreases the protein precipitated.

(b) When chondroitin sulfate is added in increasing amounts to the diluted dialyzed serum in water, the protein precipitated increases to a peak and then diminishes as shown in Figure 2, curve A. At concentrations of chondroitin sul-

TABLE I

Amounts of protein precipitable on dialysis from serum containing added chondroitin sulfate

Potassium chondroitin sulfate added	Precipitated euglobulin	Increase in euglobulin due to chondroitin sulfate added
mg.	*mg.*	*mg.*
0	19.2	0
.5	23.0	3.8
1.0	26.2	7.0
1.5	28.8	9.6

[1] This investigation was supported (in part) by research grant A-28(C) from the National Institute of Arthritis and Metabolic Diseases, United States Public Health Service, and (in part) by the Masonic Foundation for Medical Research and Human Welfare, and (in part) by the New York Chapter, The Arthritis and Rheumatism Foundation.

[2] Smith-Mundt, Fulbright Fellow, Present address: Medical Faculty, University of Paris, Paris, France.

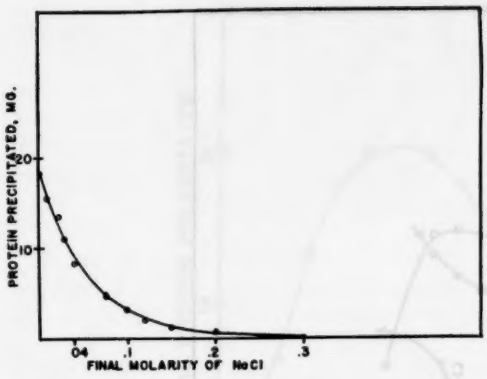


FIG. 1. PROTEIN PRECIPITATED WITH CHONDROITIN SULFATE AND DILUTED DIALYZED SERUM: EFFECT OF SODIUM CHLORIDE CONCENTRATION

Serum proteins (100 mg.) and potassium chondroitin sulfate (2 mg.) in citrate-phosphate buffer (10 ml.), .01 M Na_2HPO_4, pH 5.1.

fate over three times that causing the peak precipitation of protein, chondroitin sulfate does not precipitate any protein. At the optimal concentration, the ratio between the weight of precipitated protein and added polysaccharide is between 5 and 7.

(c) To a series of tubes, each containing 5 ml. diluted dialyzed serum in water and 0.5 ml. Na_2HPO_4 solution 0.2 M, increasing amounts of citric acid solution 0.1 M were added (0.1 to 1.5 ml.) to give a range of pH from 7.5 to 4.0. If in some tubes any precipitate appeared it was removed by centrifugation. After standing an hour, a constant amount of potassium chondroitin sulfate (1 mg.) was added to all tubes and volumes were made to 10 ml. Figure 3 shows how the amount of protein precipitated by the polysaccharide depends on the pH (curve A). Figure 2 shows how markedly the shape of the curve relating the amount of protein precipitated and the amount of potassium chondroitin sulfate added depends on pH. At pH 5.5 (Figure 2, curve B) the curve reaches a plateau, at pH 4.6 (Figure 2, curve C) there is neither a peak nor a plateau.

(d) Comparison at pH 4.6 of the precipitation curves of the globulin and the albumin fractions (separated with 55 per cent ammonium sulfate) on addition of increasing amounts of chondroitin sulfate shows that albumin produces a linear

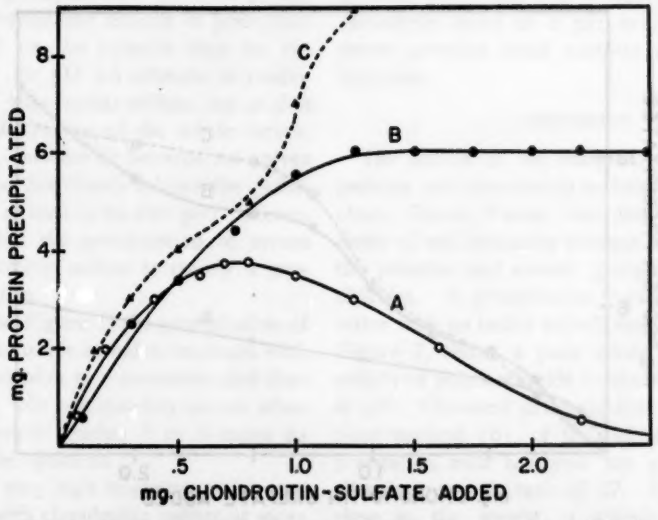


FIG. 2. PROTEIN PRECIPITATED FROM DILUTED DIALYZED SERUM (50 MG. PROTEIN IN 10 ML.) WITH INCREASING AMOUNTS OF POTASSIUM CHONDROITIN SULFATE

A, no buffer; B, citrate-phosphate buffer, .01 M Na_2HPO_4, pH 5.5; C, citrate-phosphate buffer, .01 M Na_2HPO_4, pH 4.6.

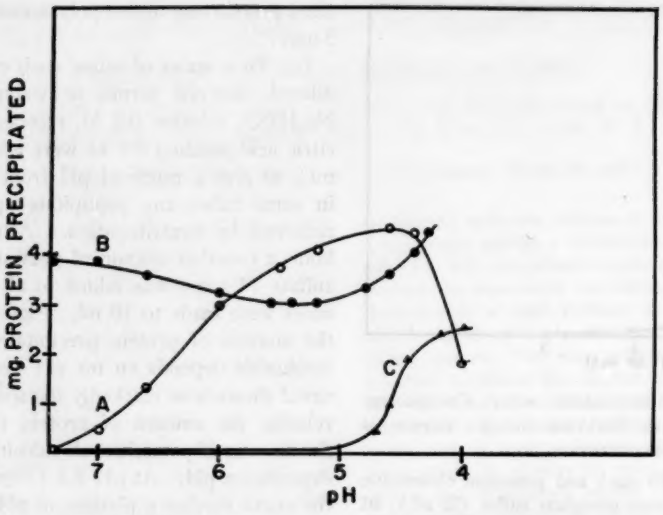


FIG. 3. PROTEIN PRECIPITATED WITH CHONDROITIN SULFATE

Each solution contained protein (50 mg.), potassium chondroitin sulfate (.5 mg.) and .01 M Na_2HPO_4 (10 ml.) to which were added increasing amounts of citric acid to vary the pH. A, proteins of dialyzed serum; B, as A, but containing in addition 15 mg. lysozyme and clearing any turbidity produced by centrifugation before adding the chondroitin sulfate; C, serum albumin, commercial.

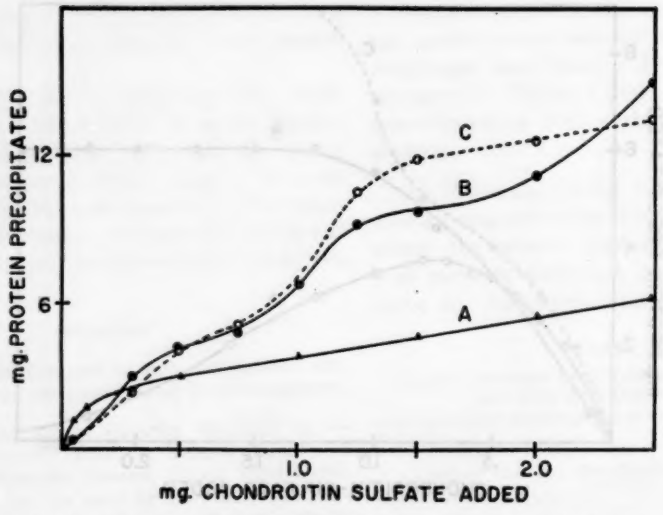


FIG. 4. PROTEIN PRECIPITATED WITH INCREASING AMOUNTS OF CHONDROITIN SULFATE

Each solution contained protein (50 mg.) in citrate-phosphate buffer (10 ml.) .01 M Na_2HPO_4, pH 4.6. A, serum albumin; B, dialyzed serum; C, serum pseudo globulin.

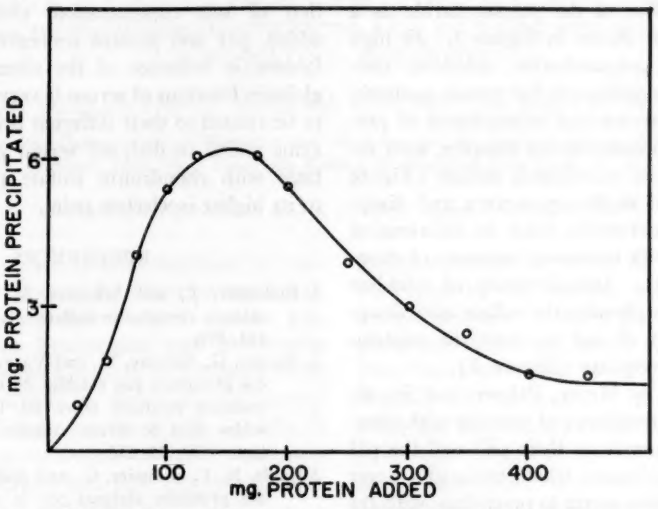


FIG. 5. PROTEIN PRECIPITATED WITH CHONDROITIN SULFATE

Effect of increasing amounts of dialyzed serum proteins added to potassium chondroitin sulfate (2 mg.) in citrate-phosphate buffer (10 ml.,) .01 M Na_2HPO_4, pH 4.6.

curve (Figure 4, curve A) quite different from that produced by the globulins (Figure 4, curve C). This latter curve has two shoulders, probably related to the precipitation of different groups of proteins. Moreover, the amount of precipitate formed is smaller for the albumin than for the globulin fraction. At pH 4.6 albumin is readily precipitable by K chondroitin sulfate, but at this pH the precipitation curve of the whole serum, Figure 4, curve B, does not lie between the curves of the two fractions, but closely follows the globulin fraction curve, at least in its first part. Hence, it seems likely that the precipitation of serum proteins by chondroitin sulfate is mainly a precipitation of globulins.

(e) As shown in Figure 5, the precipitation of the protein-polysaccharide complex increases with the protein concentration to a maximum and then decreases rapidly. No precipitation occurs when the amount of protein reaches 3 or 4 times its concentration at the optimum.

Proteins with a very high isoelectric point produce precipitates with chondroitin sulfate at more alkaline reaction than any serum proteins. For instance, lysozyme (pKi 10.5 to 11 according to Alderton, Ward, and Fevold [4]) added to a dialyzed serum diluted with a weak buffer gives a completely different precipitation curve as a function of the pH. As shown in Figure 3, curve B, precipitation remains practically at a plateau between pH 8 and 5 as if lysozyme bound polysaccharide down to a pH acid enough so the serum proteins could compete successfully with lysozyme.

DISCUSSION

The nature of the complexes between serum proteins and chondroitin sulfate is not completely clear. Meyer, Palmer, and Smyth (5) gave evidence of salt formation between cationic groups of the proteins and anionic groups of the polysaccharides. A precipitation curve of lysozyme in water with no buffer added, similar to curve A of Figure 2, shows a peak when the ratio of the weight of polysaccharide to the weight of protein is .25. Chemical determination, by the tryptophan method (6), of the polysaccharide in the precipitate with lysozyme has given a polysaccharide to protein ratio of .27. These figures are close to the weight of anionic polysaccharide equivalent to the cationic groups per gram of lysozyme (according to Fromageot [7]).

The redissolution of precipitated protein at higher concentrations of chondroitin sulfate may

be due to the action of the polysaccharide as a salt, similar to that shown in Figure 1. At high concentration of polysaccharide solutions containing protein are opalescent but remain perfectly stable. The appearance and redissolution of precipitated protein-polysaccharide complex with increasing amounts of chondroitin sulfate (Figure 2) may be related to the appearance and disappearance of metachromatic color in solutions of toluidine blue 0 with increasing amounts of chondroitin sulfate (8). Metachromasy of toluidine blue 0 induced by chondroitin sulfate also disappears on addition of salt as does the protein-polysaccharide precipitate (Figure 1).

As pointed out by Meyer, Palmer, and Smyth, the formation of precipitates of proteins with chondroitin sulfate depends on their pKi and the pH of the solution. Albumin which has a pKi lower than that of globulins starts to precipitate with the polysaccharide at pH 4.9, while globulins begin to precipitate at pH 6.9. Lysozyme which has a pKi much higher than that of globulins, precipitates with chondroitin sulfate even at pH higher than 8. The results of this study provide an experimental basis defining the conditions under which chondroitin sulfate might take part in the formation of fibrinoid, sclerotic, hyaline, and amyloid material as suggested by Altschuler and Angevine (9).

SUMMARY

Small amounts of chondroitin sulfate added to serum increase the amount of euglobulin precipitated on dialysis. The amount of precipitate produced in euglobulin-free serum by addition of chondroitin sulfate has been studied as a function of salt concentration, chondroitin sulfate added, pH and protein concentration. The difference in behavior of the albumin and pseudo globulin fractions of serum is very great and seems to be related to their different pKi values. Lysozyme added to dialyzed serum produces precipitates with chondroitin sulfate primarily related to its higher isoelectric point.

REFERENCES

1. Einbinder, J., and Schubert, M., Crystallization of calcium chondroitin sulfate. J. Biol. Chem., 1951, **191**, 591.
2. Sandor, G., Sabetay, Y., and Vargues, R., Le système des globulines peu solubles du sérum. I. Étude de quelques relations entre les lipides et les protéides dans le sérum sanguin. Bull. Soc. chim. biol., 1953, **35**, 273.
3. Jayle, M. F., Boussier, G., and Badin, J., Microdosage des protéines sériques par la méthode du biuret. Bull. Soc. chim. biol., 1951, **33**, 881.
4. Alderton, G., Ward, W. H., and Fevold, H. L., Isolation of lysozyme from egg white. J. Biol. Chem., 1945, **157**, 43.
5. Meyer, K., Palmer, J. W., and Smyth, E. M., On glycoproteins. V. Protein complexes of chondroitinsulfuric acid. J. Biol. Chem., 1937, **119**, 501.
6. Badin, J., Jackson, C., and Schubert, M., Improved method for determination of plasma polysaccharides with tryptophan. Proc. Soc. Exper. Biol. & Med., 1953, **84**, 288.
7. Fromageot, C., Lysozyme. Bull. Soc. chim. biol., 1948, **30**, No. 11–12 Suppl., 63.
8. Levine, A., and Schubert, M., Metachromasy of thiazine dyes produced by chondroitin sulfate. J. Am. Chem. Soc., 1952, **74**, 91.
9. Altshuler, C. H., and Angevine, D. M., Acid mucopolysaccharide in degenerative disease of connective tissue, with special reference to serous inflammation. Am. J. Path., 1951, **27**, 141.

PLASMA POLYSACCHARIDE FRACTION CONTAINING URONIC ACID, IN NORMAL SUBJECTS AND IN PATIENTS WITH RHEUMATOID ARTHRITIS[1]

By JACQUES BADIN,[2] MAXWELL SCHUBERT, AND MARY VOURAS

(From the Departments of Chemistry and Medicine and the Study Group on Rheumatic Diseases, New York University College of Medicine, New York, N. Y.)

(Submitted for publication March 10, 1955; accepted March 30, 1955)

Recent work on the ground substance of connective tissue has stressed the importance of mucopolysaccharide components which contain glucuronate such as hyaluronate and chondroitin sulfate. In different tissues these polysaccharides appear to vary widely in degree of polymerization and in relative quantity (1–3). Little is known about the catabolism of the ground substance polysaccharides and their possible elimination through blood and urine.

The total polysaccharide content of plasma has frequently been studied and found to contain mannose, galactose, fucose, and glucosamine (4–7). In human plasma the polysaccharides are contained mainly in the globulins (8). Albumin normally contains much less (9). Of the total polysaccharide about 10 per cent is in the mucoprotein fraction and is not precipitated by high concentration of ethanol or by 6 per cent perchloric acid (10–13). In these studies no significant amount of hexuronate has been found in any plasma polysaccharide fraction, perhaps due to the unreliability of uronic acid determination in the presence of large amounts of protein, and the drastic methods of protein hydrolysis used. The glucuronate of tungstic acid treated plasma filtrates (14) is not part of a polysaccharide. The only report of glucuronate bound to serum proteins is that of Stary and Yuvanidis (15). They precipitated serum proteins with trichloracetic acid, hydrolyzed them by boiling four hours in concentrated HCl and used Tollens' naphthoresorcinol reaction on the hydrolysates. They claimed this reaction was specific for glucuronic acid and reported good recovery of free glucuronic acid added to the precipitated protein. They found about 100 to 168 μg. glucuronic acid bound to 100 mg. protein. However they did not study the recovery of added polysaccharide such as chondroitin sulfate and did not point out that the color developed in a serum protein hydrolysate differed from that developed by glucuronic acid. In the present work, difficulties, to be described later, were found with Tollens' method when applied to plasma fractions containing polysaccharides.

Polysaccharides of urine, on the contrary, do contain glucuronate. Tayeau, Biserte, Montreuil, and Marqueville (16) reported a mucoprotein in urine containing mannose, fucose, galactose, acetylhexosamine and hexuronic acid. Kerby (17) found a polysaccharide in urine which contained hexuronic acid and hexosamine and which on paper electrophoresis behaved as chondroitin sulfate. This polysaccharide was not derived from the urinary tract or the prostatic gland.

In the present work a method has been developed which separates from plasma a material which contains polysaccharide with glucuronic acid as a component. In this form the glucuronic acid can be quantitatively determined. The method involves two novel procedures. The first is based on the previous observation (18) that chondroitin sulfate added to plasma is recovered in the euglobulin fraction. The glucuronate-containing material of plasma is also found entirely concentrated in the euglobulin fraction, since examination of the euglobulin-free plasma revealed no glucuronate. The second new procedure is the separation, after protein digestion, of the glucuronate-containing material by the relatively

[1] This investigation was supported (in part) by research grant A-28(C) from the National Institute of Arthritis and Metabolic Diseases, of the National Institutes of Health, U. S. Public Health Service, and (in part) by the Masonic Foundation for Medical Research and Human Welfare and the New York Chapter, The Arthritis and Rheumatism Foundation.

[2] Smith-Mundt, Fulbright Fellow. Present address: Medical Faculty, University of Paris, France.

selective action of lysozyme in precipitating high molecular weight anionic materials.

EXPERIMENTAL

The hexuronate-containing fraction of plasma was isolated in the following six steps.

1) Blood (30 to 40 ml.) was collected with addition of sodium citrate (1 ml., 10 per cent solution). The sample was centrifuged, and the plasma separated and diluted with 3 vol. of aqueous sodium citrate (0.1 per cent).

2) Using Sandor, Sabetay, and Vargues' procedure (19) the diluted plasma was dialyzed in a cellophane bag against 2 L. of buffer (citric acid, Na_2HPO_4, M/150, pH 5.8 ± .1). After dialysis for 3 days at 4° C. euglobulins were centrifuged and then washed with 5 ml. buffer, then with ethanol and ether, dried and weighed (column 3, Table I).

3) The only suitable digestion procedure found was as follows. A solution of crystalline pepsin (3 mg. per ml. in .1 M HCl) was added to the protein in the ratio of 6 mg. pepsin for 100 mg. protein. Samples were gently shaken at room temperature for 3 days and then neutralized with 1 M NaOH. A solution of crystalline trypsin (3.5 mg. per ml. in HCl-sodium barbital buffer, M/7, pH 8.4) was then added to give 2 mg. trypsin for 100 mg. original protein. This gave a pH of about 8 and a sodium barbital molarity of about M/35. Toluene (1 ml.) was added and digestion continued 3 days at about 30° C.

4) The hydrolysates were neutralized with acetic acid and made up to 10 ml. with water. Perchloric acid, 60 per cent, was added to give a final concentration of 6 per cent. After standing an hour, precipitated proteins were centrifuged, washed with ethanol, dissolved in 1 ml. alkaline saline and protein determined with the biuret reaction. Deduction of this residual protein from the initial sample gave the amount of protein digested (column 4, Table I).

5) The solution of protein hydrolysate containing $HClO_4$ was dialyzed in cellophane bags against 3 L. potassium acetate solution, 0.1 per cent, for 24 hours and then added to 10 vol. of a mixture of ethanol (8 parts) and ether (2 parts) containing potassium acetate (0.5 per cent) in flasks. After 48 hours at room temperature the polysaccharides adhered to the glass and the fluid was poured off. Flasks were dried 1 hour at 40° C. and polysaccharides were dissolved in 10 ml. water.

6) To each solution was added a freshly made, clear solution of lysozyme, 15 mg. in 5 ml. water. Turbidity appeared immediately and a flocculent precipitate soon formed which was centrifuged easily after 3 hours and washed with water.

The material precipitated by lysozyme was dissolved in 1 ml. saline alkalinized with a trace of NaOH and used for the carbazole test as described below. All samples developed the characteristic pink color as did glucuronic acid. Seven samples of normal human euglobulins gave an average of 57 μg. glucuronic acid for 100 mg. protein digested (column 5, Table I). Recovery of

TABLE I

Results of analysis, by the procedure described, of the euglobulin of plasma of normals and of subjects with rheumatoid arthritis

	Euglobulins			Hexuronic acid	
	Per 100 ml. plasma (mg.)	Used for determ. (mg.)	Digested by enzyme (mg.)	Per 100 mg. euglobulin (μg.)	Per 100 ml. plasma (μg.)
Rheumatoid subjects					
1. Ben.	431	95	92	75.0	323
2. Der.	905	145	137	58.0	525
3. Ga.	393	51	48	152.0	597
4. In.	785	126	120	51.7	406
5. Po.	694	111	106	53.8	373
6. Rot.	540	54	51	68.6	370
7. Wo.	533	96	92	67.5	359
8. Av.	1,470	280	276	23.1	339
9. Man.	500	100	98	34.6	173
10. Mas.	1,210	230	221	34.1	412
Average	746			61.8	388
Normals					
1. No. 3934	384	300	295	44.8	172
2. MacL.	430	300	294	35.7	153
3. Cap.	331	133	126	66.7	221
4. Ken.	340	137	130	54.1	184
5. Cruz.	425	162	154	65.0	276
6. Sant.	340	137	130	46.2	157
7. Sla.	325	131	124	86.5	281
Average	361			57.0	206

potassium chondroitin sulfate (1 to 5 mg.) added to the original protein samples was between 66 and 100 per cent.

The proteins of the diluted plasma, which remained after removal of the euglobulins, were precipitated with ten volumes of ethanol and then treated by the six steps described. In no case did the products develop the characteristic pink color in the carbazole reaction.

For further characterization, larger amounts of the product containing hexuronic acid were required. The same procedure was used starting with 1 to 2 gm. euglobulin. The lysozyme precipitate was centrifuged and dissolved in 5 ml. alkalinized saline. On this larger sample glucuronic acid, total polysaccharides, hexosamine and protein could be determined.

ANALYTICAL METHODS

Tollens' naphthoresorcinol method has been considered to be specific for the determination of glucuronic acid (20). In this test a deep violet color develops in the ether layer whose intensity is proportional to the amount of glucuronic acid. The method as described by Ratish and Bullowa (21) was found unsuitable for our purpose. Addition of chondroitin sulfate to serum protein gave less than 50 per cent recovery of hexuronic acid. From plasma protein hydrolysates, which contain large quantities of other sugars, the colored complex was not extracted with ether. In the presence of added hexoses, complexes seemed to be formed with naphthoresorcinol and hexuronic acid which were not soluble in ether. By adding pyridine, these complexes became soluble in ether and a deep purple color developed which was the sum of the colors due to glucuronic acid and the added hexoses. Attempts to separate the violet complex due to glucuronic acid from the purple complexes due to other sugars by chromatography using toluene and toluene-glycol systems failed.

The carbazole reaction of Dische (22), on the contrary, was found very sensitive and specific for uronic acids. This reaction did not give good results on biological materials since some amino acids and high concentrations of serum lipids interfere with the development of color. Mannose, galactose, fucose, and glucose do not give a pink color, as do hexuronic acids, but rather a pale brown-yellow color with a completely different absorption spectrum (Figure 1). This reaction has been considered to measure uronic acids only when the color developed was bright pink or purple with a peak at 520 mμ. For determinations on polysaccharide-lysozyme complexes it was found important to have controls in the presence of a quantity of lysozyme similar to that in the precipitates. Figure 2 shows the standard curve for glucuronic acid and its modification by added lysozyme. Lysozyme when heated alone with H_2SO_4 developed a faint brown-yellow tint that deepened slightly on addition of carbazole but never turned pink. This color limits the sensitivity of the method but never corresponds to more than 5 μg. of glucuronic acid. The Dische reaction was carried out as follows.

To a sample (10 to 120 μg. uronic acid in 1 ml.) in a calibrated Klett tube, 6 ml. H_2SO_4 were added dropwise, with shaking in an ice bath; the fluid was thoroughly mixed by bubbling air through it with a fine pipette. The tubes were heated for 30 minutes in a boiling water bath, then chilled in ice water. The brownish color which developed was read with a Klett-Sumerson photometer (filter S540) and was usually less than 60 divisions. If the reading was greater, the mixtures were diluted with a solution (H_2SO_4 6 vol., water 1 vol.) to make the reading less than 60, and then read. Carbazole (0.2 ml., 0.1 per cent in alcohol) was added to each tube and the fluids mixed by bubbling air through them. The carbazole used was twice crystallized from ethanol and sublimed *in vacuo*. The tubes were set at 4° C. for 12 to 15 hours, then a second set of readings was made and the first set deducted. Samples not developing the characteristic pink color were considered as totally lacking in hexuronic acid.

For hexosamine Shetlar, Foster, Kelly, and Everett's micro method (23) was modified. The sample to be analyzed (1 ml.) was hydrolyzed 4 hours with HCl (1 ml., 8M) at 100° C. The mixture was decolorized with Darco (50 mg.), centrifuged, the Darco washed with water and the washings added to the hydrolysate. The method, continued as described by Shetlar, allowed determination of 20 to 100 μg. hexosamine with about 10 to 15 per cent error.

Total sugars determined with the modified tryptophane method (24, 25) gave results with an error of about 10 per cent.

Proteins estimated with the biuret method were reliable when solutions were colorless and no turbidity developed with the reagent (26).

The larger samples of precipitate obtained with

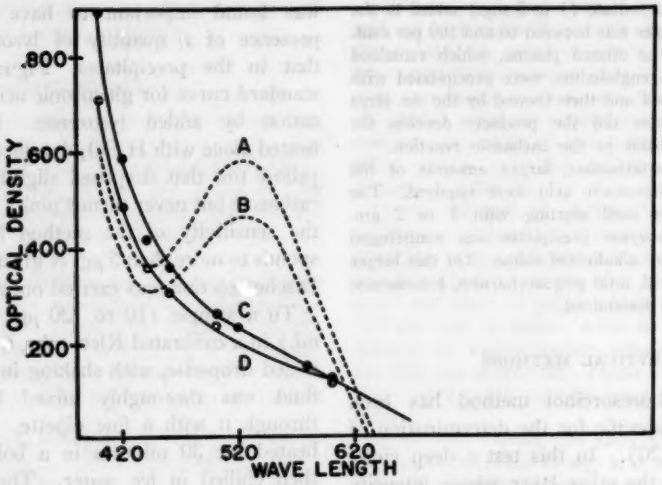


FIG. 1. SPECTRA OF CARBAZOLE REACTION PRODUCTS IN SULFURIC ACID WITH DIFFERENT SUGARS

A, galacturone (60 μg.); B, glucurone (50 μg.); C, mannose (100 μg.); D, galactose (100 μg.). Glucose, glucosamine, and fucose give spectra almost identical with those of mannose and galactose.

lysozyme from 1 to 2 gm. of normal human euglobulin as described above were analyzed for hexuronate, hexosamine, total sugars, and protein. The analytical results were subjected to several corrections. The lysozyme (Worthington Biochemical Corp.) was similarly analyzed and found to contain 164 μg. total sugars and 52 μg. hexosamine per 100 mg., but no detectable

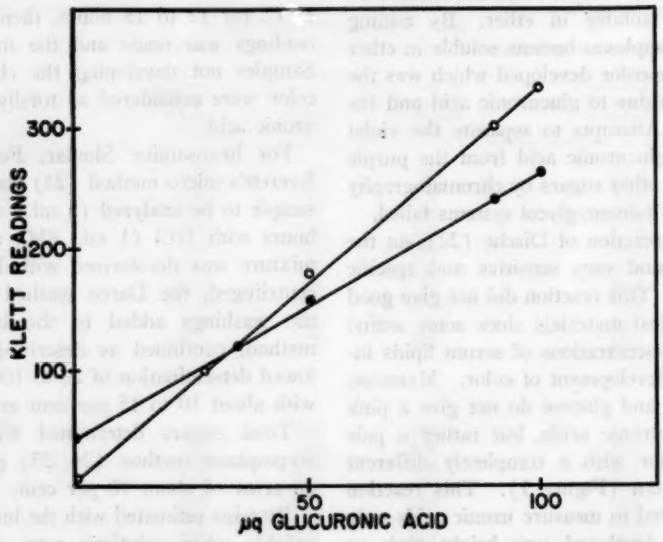


FIG. 2. INFLUENCE OF LYSOZYME ON DISCHE'S CARBAZOLE REACTION

○, glucuronic acid; ●, glucuronic acid in the presence of a constant amount (5 mg.) of lysozyme.

TABLE II

Results of analysis of material precipitated with alcohol and the polysaccharide material precipitated with lysozyme *

Starting materials (1 gm.)†	Total sugars mg.	Hexuronic acid mg.	Hexosamine mg.	Protein (Biuret) mg.
Total polysaccharides precipitated in step 5				
Euglobulins	13.50	No pink color develops‡	7.35	42.0
Lysozyme precipitates produced in step 6				
Euglobulins	.408	.480	.483	20.8
Total plasma proteins (including euglobulins)	.043	.072	.191	14.4
Total plasma proteins with added chondroitin sulfate (1.33 mg.)		.342	.400	17.5
Lysozyme precipitate of potassium chondroitin sulfate carried through steps 1–6				
Chondroitin sulfate (1 mg.)		.270	.209	
Calculated		.266	.250	

* No attempt was made to calculate the percentages of the several components since the products were never isolated in a dry form.

† Euglobulins were prepared as in steps 1 and 2. Total plasma proteins were prepared by the addition to plasma of ten volumes of ethanol, and the product was washed with ethanol and ether.

‡ The failure of a pink color to develop in this material, although glucuronic acid must be present, is probably due to the presence of the large excess of hexoses and also a larger proportion of protein.

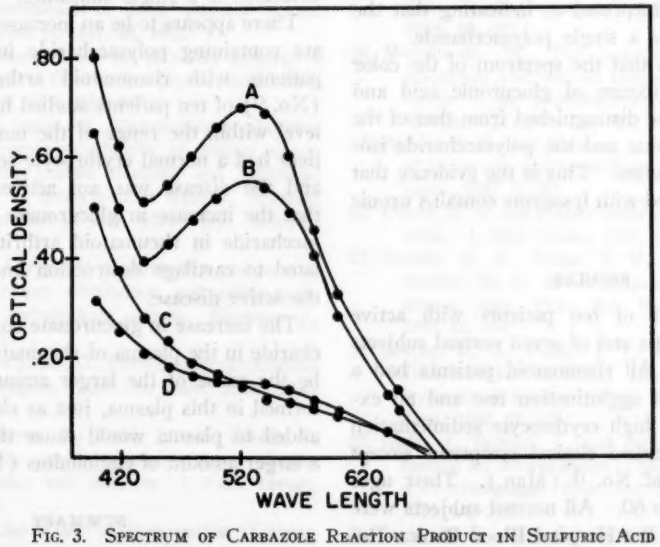


FIG. 3. SPECTRUM OF CARBAZOLE REACTION PRODUCT IN SULFURIC ACID WITH THE ACID POLYSACCHARIDE FROM SERUM EUGLOBULINS AND SOME CONTROLS

A, euglobulin polysaccharide precipitated with lysozyme; B, 5 μg. of glucurone and 8 mg. of lysozyme; C, 8 mg. of lysozyme; D, polysaccharide from dialyzed serum (without euglobulins) and 8 mg. of lysozyme. Spectra measured with Beckman spectrophotometer.

hexuronate. Estimations on lysozyme-polysaccharide precipitates were corrected by deducting the amounts of sugar and hexosamine present in the quantity of lysozyme developing the same color with the biuret reaction. The color of the tryptophane reaction for total polysaccharides is due to hexoses, pentoses, and uronic acids. To measure the sugars, the color corresponding to uronic acids was deducted, 100 μg. of glucuronic acid developing color corresponding to 70 μg. mannose (25). Table II contains data on analysis of the material precipitated with lysozyme in step 6. If the total polysaccharides of step 5 are examined with the Dische reaction, no glucuronic acid can be detected because the pink color of the carbazole reaction does not develop with this product. Only after the selective precipitation with lysozyme in step 6 does hexuronic acid appear in the Dische reaction with the characteristic pink color. The euglobulin fraction was found to contain all the hexuronic acid of the total plasma proteins. The figures for the material isolated from euglobulins with lysozyme show the following proportions: 1.00 mol. hexuronic acid, 1.09 mol. hexosamine, .91 mol. sugar. These figures cannot be interpreted as indicating that the product isolated is a single polysaccharide.

Figure 3 shows that the spectrum of the color produced by a mixture of glucuronic acid and lysozyme cannot be distinguished from that of the complex of lysozyme and the polysaccharide isolated from euglobulins. This is the evidence that the product isolated with lysozyme contains uronic acid.

RESULTS

Plasma samples of ten patients with active rheumatoid arthritis and of seven normal subjects were compared. All rheumatoid patients had a positive sheep cell agglutination test and all except No. 9 had a high erythrocyte sedimentation rate. All had marked clinical symptoms except No. 1 (Ben.) and No. 9 (Man.). Their ages ranged from 30 to 60. All normal subjects were healthy donors at the Hospital Blood Bank. Results in Table I show no difference in the glucuronic acid calculated per 100 mg. euglobulins between normals and rheumatoids (column 5, Table I). When calculated per 100 ml. of plasma (column 6, Table I) there seems to be a difference; all but one of the patients had a higher plasma polysaccharide glucuronate level than the normals.

DISCUSSION

The presence of the glucuronate containing polysaccharide exclusively in the euglobulin fraction is an expression of its marked anionic properties. It behaves as free chondroitin sulfate does when added to plasma *in vitro* (18). Dried on a glass slide the lysozyme complex, prepared as described from euglobulin, gives with toluidine blue 0 the characteristic deep violet metachromatic color. Lysozyme alone does not do so. The identity of the material isolated from the euglobulin is not certain. It has an unexpectedly large protein component. Table II shows that the ratio of protein to combined sugar is about 15 in the lysozyme precipitate derived from euglobulin while in the precipitate produced by lysozyme and chondroitin sulfate the ratio is about 5 (18). The material isolated from euglobulin appears to contain protein not completely removed by enzyme digestion. There is no evidence that the material described is a single substance.

There appears to be an increase in the glucuronate containing polysaccharide in the plasma of patients with rheumatoid arthritis. Only one (No. 9) of ten patients studied had a glucuronate level within the range of the normals. This patient had a normal erythrocyte sedimentation rate and the disease was not active. It is possible that the increase in glucuronate containing polysaccharide in rheumatoid arthritis plasma is related to cartilage destruction known to occur in the active disease.

The increase in glucuronate containing polysaccharide in the plasma of rheumatoid arthritis may be the cause of the larger amount of euglobulin formed in this plasma, just as chondroitin sulfate added to plasma would cause the appearance of a larger amount of euglobulins (18).

SUMMARY

1. A material behaving as an anionic polysaccharide containing uronic acid, hexosamine and one or more sugars has been found in human euglobulin. The components are in the ratio, 1.09 mol. hexosamine, 1.00 mol. glucuronic acid,

.91 mol. sugar. No trace of this material has been found in euglobulin-free plasma.

2. An increase in this material has been found in the plasma of ten patients with rheumatoid arthritis. This increase appears as an increased amount of euglobulin and not as euglobulin with an increased amount of polysaccharide.

REFERENCES

1. Partridge, S. M., The chemistry of connective tissues. 1. The state of combination of chondroitin sulphate in cartilage. Biochem. J., 1948, **43**, 387.
2. Gersh, I., Ground substance and the plasticity of connective tissues. Harvey Lectures, 1949–50, **45**, 211.
3. Meyer, K., The mucopolysaccharides of the interfibrillar substance of the mesenchyme. Ann. New York Acad. Sc., 1950, **52**, 943.
4. Sørensen, M., and Haugaard, G., Über die Anwendbarkeit der Orcinreaktion zur Bestimmung der Art und Menge von Kohlen-hydratgruppen in Eiweissstoffen. Biochem. Ztschr., 1933, **260**, 247.
5. Rimington, C., (a) The isolation of a carbohydrate derivative from serum-proteins. Biochem. J., 1929, **23**, 430; (b) The carbohydrate complex of the serum proteins. II. Improved method for isolation and redetermination of structure. Isolation of glucosaminodimannose from proteins of ox blood. Biochem. J., 1931, **25**, 1062.
6. Bierry, H., Recherches sur les protéides sanguins à l'état normal et à l'état pathologique. Bull. Acad. de méd., Paris, 1943, **127**, 447.
7. Dische, Z., and Osnos, M., Distribution of the protein-bound mucopolysaccharide of human serum among various protein fractions and individual variations of its methylpentose content. Abstract of paper presented at Am. Chem. Soc. Meeting, March, 1953.
8. Hewitt, L. F., Chemistry of antibodies and serum-proteins. Nitrogen distribution and amino-acids. II. Protein carbohydrate groups. Biochem. J., 1934, **28**, 2080.
9. Jayle, M.-F., and Judas, O., Formule glycoprotéidique du plasma sanguin. Helv. et. chim. Acta, 1946, **29**, 1310.
10. Winzler, R., Devor, A., Mehl, J., and Smyth, I., Studies on the mucoproteins of human plasma. I. Determination and isolation. J. Clin. Invest., 1948, **27**, 609.
11. Schmid, K., Isolation and characterization of glycoproteins from human plasma. J. Am. Chem. Soc., 1953, **75**, 2532.
12. Shetlar, M. R., Payne, R. W., Bullock, J. A., Patrick, D. R., Hellbaum, A. A., and Ishmael, W. K., Comparative studies of serum polysaccharides in rheumatoid arthritis and degenerative joint disease. J. Clin. Invest., 1953, **32**, 1208.
13. Badin, J., and Glyn, J., Relationship between plasma mucoproteins and protein sugar in patients with rheumatoid arthritis receiving cortisone. Proc. Soc. Exper. Biol. & Med., 1954, **86**, 150.
14. Fashena, G. J., and Stiff, H. A., On the nature of the saccharoid fraction of human blood. II. Identification of glucuronic acid. J. Biol. Chem., 1941, **137**, 21.
15. Stary, Z., and Yuvanidis, M., Über den Hexuronsäuregehalt der Serumproteine. Biochem. Ztschr., 1953, **324**, 206.
16. Tayeau, F., Biserte, G., Montreuil, J., and Marqueville, S., Identification de la substance responsable du phénomène d'obstacle de Donaggio. Compt. rend. Acad. d. sc., 1953, **237**, 208.
17. Kerby, G., The excretion of glucuronic acid and of acid mucopolysaccharides in normal human urine. J. Clin. Invest., 1954, **33**, 1168.
18. Badin, J., and Schubert, M., Conditions of formation of euglobulin-like precipitates from serum proteins and chondroitin sulfate. J. Clin. Invest., 1955, **34**, 1312.
19. Sandor, G., Sabetay, Y., and Vargues, R., Le système des globulines peu solubles du sérum. I. Étude de quelques relations entre les lipides et les protéides dans le sérum sanguin. Bull. Soc. chim. Biol., 1953, **35**, 273.
20. Mozolowski, W., The quantitative estimation of glycuronic acid and its conjugated compounds by means of the naphthoresorcinol test of Tollens. Biochem. J., 1940, **34**, 823.
21. Ratish, H. D., and Bullowa, J. G. M., Determination of glucuronic acid in biological media. Arch. Biochem., 1943, **2**, 381.
22. Dische, Z., A new specific color reaction of hexuronic acids. J. Biol. Chem., 1947, **167**, 189.
23. Shetlar, M. R., Foster, J. V., Kelly, K. H., and Everett, M. R., Serum polysaccharide level in the normal state. Proc. Soc. Exper. Biol. & Med., 1948, **69**, 507.
24. Shetlar, M. R., Foster, J. V., and Everett, M. R., Determination of serum polysaccharides by the tryptophane reaction. Proc. Soc. Exper. Biol. & Med., 1948, **67**, 125.
25. Badin, J., Jackson, C., and Schubert, M., Improved method for determination of plasma polysaccharides with tryptophane. Proc. Soc. Exper. Biol. & Med., 1953, **84**, 288.
26. Jayle, M.-F., Badin, J., and Boussier, G., Microdosage des protéines sériques par la méthode du biuret. Bull. Soc. chim. biol., 1951, **33**, 881.

THYROXINE-BINDING CAPACITY OF SERUM IN NORMAL MAN[1]

By JACOB ROBBINS[2] AND J. E. RALL[3]

(From the Sloan Kettering Institute for Cancer Research, Memorial Center for Cancer and Allied Diseases, New York, N. Y.)

(Submitted for publication February 18, 1955; accepted April 20, 1955)

The presence of a specific thyroxine-binding protein in human serum was first suggested from studies by Gordon, Gross, O'Connor, and Pitt-Rivers (1), employing zone electrophoresis. Although such a protein has not yet been isolated, certain of its properties may be determined in unfractionated serum when the specific protein has been labeled with thyroxine-I^{131}. In this way, it has been demonstrated that this protein has: 1) an electrophoretic mobility at pH 8.6 (1–5), as well as at pH 6 and 7.6 (5), intermediate between alpha-1 and alpha-2 globulins, 2) an electrophoretic mobility at pH 4.5 similar to the "M–2 proteins," or alpha-2 glycoproteins, of serum (6), and 3) a sedimentation rate in the ultracentrifuge of approximately 3.3 Svedbergs (7).

In the present study, this technique has been applied to an investigation of the thyroxine-binding capacity of this specific protein.

MATERIALS AND METHODS

Serum was obtained after an overnight fast from six normal young adults—three men[4] and three women; from one young woman with Graves' disease; and from one young man with cretinism, who was being maintained in a euthyroid state by triiodothyronine administration. The serum was stored in the frozen state until used.

Synthetic L-thyroxine, labeled with I^{131},[5] was diluted with appropriate quantities of *n*-butanol, and evaporated to dryness *in vacuo* without heating. Serum was then added, so as to produce a wide range of thyroxine concentrations in the serum from each subject. The serum-thyroxine mixtures were allowed to stand for 15 to 20 hours at 4° C. and then were stored in the frozen state. The concentration of thyroxine in the serum-thyroxine mixtures was determined by analysis of total iodine in the synthetic thyroxine preparations, and of protein-bound iodine in the original serum, by a modification of the method of Barker (8). (It was assumed that all of the iodine so measured was in the form of thyroxine.) Recovery of labeled thyroxine after drying was assessed by comparing the radioactivity in each serum-thyroxine mixture with that in a portion of the original butanol solution. These measurements were performed with a well-type scintillation counter. The recoveries were usually between 85 and 100 per cent, but occasional values as low as 30 per cent were obtained. The latter occurred at very low dilution of the original thyroxine solution, and may be related to the fact that this original material contained a greater solid residue after evaporation than did the butanol added as diluent. Thus, this factor was present only at the highest thyroxine concentrations.

A total of 9 experiments were performed on the six normal sera, using 6 separate batches of thyroxine-I^{131}. In all, 64 individual determinations of thyroxine-binding were done. The endogenous serum thyroxine levels varied from .05 to .08 microgram per ml., and the total thyroxine levels varied from .06 to 8.8 micrograms per ml.

In order to obtain data for serum thyroxine levels lower than normal, the serum from a cretin was employed. This patient had been maintained on daily doses of 0.2 mg. of triiodothyronine for the preceding 2 months, so that his serum protein-bound iodine level was low

[1] This work was supported by a grant from the Damon Runyon Memorial Fund for Cancer Research, a grant from the American Cancer Society, and a contract with the Atomic Energy Commission, No. AT(30-1)-910.

[2] Present address: National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland.

[3] Alfred P. Sloan Foundation Scholar.

[4] One subject had ulcerative colitis, and had received a trial course of diiodoquine therapy, which had been stopped 3 months before the serum was obtained. This was discovered in retrospect, when a serum protein-bound iodine value of 19 micrograms per 100 ml. was found. The serum thyroxine level which was used in the calculations was determined by a repeat iodine determination on serum obtained 2½ months later, at which time the serum protein-bound iodine was 4.3 micrograms per 100 ml. The data of this experiment are included since they did not differ significantly from those in other experiments.

[5] L-thyroxine was obtained through the courtesy of Smith, Kline and French Laboratories, Philadelphia, Pa., and labeled with I^{131} by Abbott Laboratories, Oak Ridge, Tenn. These preparations were dissolved in n-butanol, and had specific activities of approximately 8 to 40 mc. per mg. In one instance, thyroxine-I^{131} was obtained in 50 per cent propylene glycol, and was added directly to the serum after appropriate dilution with water.

(.009 microgram per ml.) even though he was in a euthyroid state by all other laboratory and clinical criteria. For the purposes of calculation, the serum protein-bound iodine was considered to be thyroxine, although it was perhaps more likely triiodothyronine. In this serum, the total thyroxine level was adjusted to from .017 to 3.2 micrograms per ml. If the endogenous serum iodine were neglected, only the lowest thyroxine values would be affected, and, even there, the general form of the results would be unaltered.

The serum from the hyperthyroid patient was used to evaluate the degree of exchange between endogenous serum thyroxine and added synthetic thyroxine (see Results).

The purity of the thyroxine-I^{131} preparations was evaluated by paper chromatography with techniques described elsewhere (4), except that a continuously recording counting rate meter was used (see below). Each batch was chromatographed in a *n*-butanol—2,4-dioxane—2N ammonia system (9), and some in a n-butanol—primary amyl alcohol—2N ammonia system (4) as well. In the 7 batches of labeled thyroxine used in this study, thyroxine-I^{131} comprised from 72 to 91 per cent (mean = 83 per cent) of the total radioactivity. From 2 to 7 per cent of the total radioactivity behaved like iodide; and the remainder was due to one or more contaminants of unknown nature, with Rf values higher than thyroxine. These contaminants did not appear to be triiodothyronine or diiodothyronine. Chromatographic analyses of the thyroxine-serum mixtures [6] were identical with those of the original butanol solutions, with the exception of from 1 to 4 per cent immobile radioiodine in the former.

The thyroxine concentration measurement was not corrected for this impurity factor, since the behavior of the major contaminants with regard to protein-binding has not been determined. In two experiments with normal sera, and in one with hyperthyroid serum, thyroxine-I^{131} was kept constant, while the total thyroxine was varied by adding unlabeled L-thyroxine. The purity of this material was 97 per cent, according to analyses in the Smith, Kline, and French Laboratories.[7]

Each serum-thyroxine mixture was studied by zone electrophoresis in filter paper at pH 8.6 (barbital buffer, ionic strength 0.1), by techniques described elsewhere (4, 6). The distribution of radioiodine among the various serum protein components was determined by counting the strips with a thin mica-window Geiger-Mueller tube and a continuously recording counting rate meter. The counting aperture was a 1×2.7 cm. rectangle. Since the area under the curves obtained with the counting apparatus was proportional to the quantity of radioactivity on the paper strip, the amount of I^{131} in the protein fractions could be measured by planimetry. In 2 experiments, the strips were stained prior to counting. Since this procedure resulted in a slight underestimation (approximately 10 per cent) of radioiodine in alpha globulin, due to a relatively greater loss of this component during washing, all subsequent radioiodine measurements were performed prior to staining.

In the results which follow, total serum thyroxine concentration represents the sum of endogenous and added thyroxine. Thyroxine bound to alpha globulin, or to albumin, was calculated as the product of total serum thyroxine and the fraction of total serum radioiodine associated with the protein in the electrophoretic analyses.

RESULTS

Figure 1 illustrates the electrophoretic patterns obtained at various serum thyroxine levels. At a normal level (0.10 microgram per ml.), the major portion of the thyroxine-I^{131} has a mobility intermediate between alpha-1 and alpha-2 globulin, but overlaps both of these components. A smaller portion coincides with albumin, and a still smaller amount, with the other protein fractions. At least part of the radioiodine associated with the beta and gamma globulins probably represents adsorption of albumin or alpha globulin radioiodine on the paper, as suggested by the presence of radioiodine between the point of application and

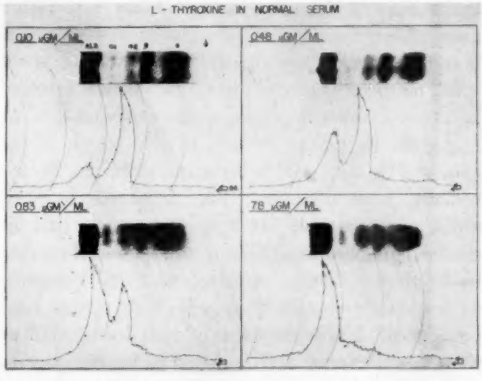


FIG. 1. THE EFFECT OF INCREASING CONCENTRATIONS OF SERUM THYROXINE ON THE ELECTROPHORETIC DISTRIBUTION OF THYROXINE-I^{131}

Synthetic thyroxine-I^{131} was added to normal serum, producing the total serum thyroxine levels indicated in the figure. In each instance, a continuous recording of the radioactivity on the paper strip (lower portion), is compared with the stained protein components on the same paper strip (upper portion). The single arrow denotes the point of application of the serum to the paper, and the double arrow, labeled "BG," represents background radioactivity. (Buffer: sodium barbital, pH 8.6, ionic strength 0.1.)

[6] The thyroxine-serum mixtures were applied directly to the paper strips without prior extraction (4).

[7] The authors wish to thank Dr. A. Heming of the Smith, Kline, and French Laboratories for these analyses.

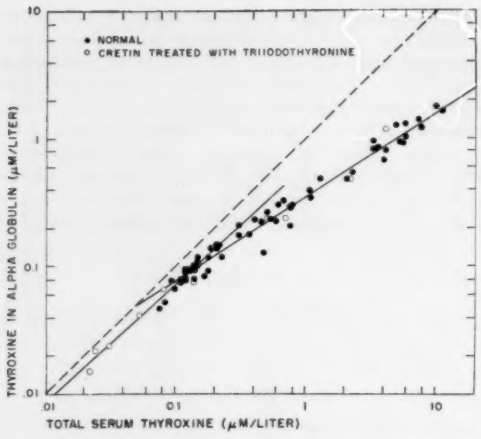


FIG. 2. THE CONCENTRATION OF ALPHA GLOBULIN-BOUND THYROXINE AT INCREASING CONCENTRATIONS OF TOTAL SERUM THYROXINE

● = normals, ○ = cretin treated with triiodothyronine. See text for the procedure by which the solid lines were fitted to the experimental data. The broken line represents 100 per cent binding of serum thyroxine to alpha globulin.

the first protein band. A small band of radioiodine also is present with a mobility just faster than albumin. This is thought to represent an impurity in the thyroxine-I^{131} preparations, since it has not been observed in sera in which thyroxine has been labeled by endogenous synthesis.

As the thyroxine content of the serum is increased, thyroxine-I^{131} becomes increasingly associated with albumin. The proportion in the alpha globulin area becomes less, while that in the beta and gamma globulin areas, as well as that in front of albumin, remains relatively constant. In some experiments at very high thyroxine concentrations, larger amounts of radioiodine (15 to 35 per cent of total I^{131}) were found in the gamma globulin, area, and, especially, in the form of a rather sharp band at the point of application. This appeared to represent thyroxine displaced from albumin during electrophoresis, since in these experiments the albumin thyroxine contents were somewhat lower. Free thyroxine would be expected to behave in this way (1).

Although an excessive proportion of immobile plus gamma globulin thyroxine-I^{131} (more than 15 per cent of total I^{131}) was found in only 6 of the 12 experiments, other evidence was sought with regard to the possibility of incomplete protein-binding at high thyroxine levels. Analysis in the ultracentrifuge partition cell [8] (7) indicated, however, that essentially all of the added thyroxine was bound to protein at even the highest thyroxine concentrations. The excessive immobile plus gamma globulin thyroxine-I^{131} did, therefore, appear to result from displacement of thyroxine from protein—presumably albumin.

Over the normal range of serum thyroxine concentration (.059 to 0.12 microgram per ml., or .076 to 0.15 micromole per liter), the values for per cent of total radioiodine in alpha globulin varied in an apparently random fashion between the limits of about 60 and 80 per cent. At thyroxine levels greater than 0.27 microgram per ml. (0.35 micromole per liter), all of the values were below 60 per cent and fell to a minimum of 14 per cent at the highest thyroxine levels tested. The exact point of transition, which would reflect that concentration of thyroxine at which the TBP is saturated, could not, however, be delineated from a plot of per cent of I^{131} in alpha globulin *versus* total serum thyroxine. At thyroxine levels below the normal range the per cent in alpha globulin was somewhat higher than in the normal range, but these values were all obtained in a single abnormal individual, and the low levels of radioactivity in these thyroxine-serum mixtures made the electrophoretic analyses less accurate. A definite radioactive peak in albumin was, however, present at even the lowest concentration.

In Figure 2, the concentration of thyroxine in alpha globulin in the normal sera, and in the cretin under triiodothyronine therapy, are plotted against total serum thyroxine on logarithmic coordinates. The data presented are not classified according to the individual experiments (see Materials and Methods), since there appeared to be no significant variation between the various sera or batches of thyroxine. The serum from the cretin, however, is distinguished from the normals. It is apparent that the concentration of thyroxine in alpha globulin continues to increase throughout the range employed. The data, however, appear to fall into two linear components.

[8] The authors are indebted to Dr. Mary L. Petermann, of the Sloan Kettering Institute, New York, N. Y., for these determinations.

TABLE I

Statistical evaluation of data in figure 2

Grouping	Separation point—Total serum thyroxine	No. in lower group	No. in upper group	Equations for lines	Intersection value—Total serum thyroxine	.05 Confidence limits of intersection value	
						Lower	Upper
	(μm/L.)				(μm/L.)	(μm/L.)	(μm/L.)
1st	.250	34	40	Log Y = .645 Log X − .4526 Log Y = .895 Log X − .2576	.166	.0342	.600
2nd	.166	26	48	Log Y = .647 Log X − .4533 Log Y = .927 Log X − .2182	.145	.0947	.241
3rd	.145	23	51	Log Y = .643 Log X − .4799 Log Y = .916 Log X − .2326	.124	.104	.290
4th	.124	15	59	Log Y = .651 Log X − .4546 Log Y = .942 Log X − .1981	.132	.0724	.852

These two groups were divided arbitrarily at a total serum thyroxine level of 0.25 micromole per liter, and the data for each section were fitted, by least squares, to the best straight line having the form log Y = A + B log X (where Y = thyroxine concentration in alpha globulin, X = total serum thyroxine concentration). These lines intersected at a total serum thyroxine concentration of 0.166 micromole per liter. The points were then regrouped according to this value, and the process of fitting the data to two straight lines were repeated. Two additional regroupings were done on the basis of the new intersections. The intersections and the 95 per cent confidence levels of the total serum thyroxine values of the intersections are recorded in Table I (10). This method of successive approximation removed the main subjective portion of the statistical treatment. The 95 per cent confidence level of the second intersection, measured by X, lay between .0947 and 0.241 micromole per liter. It seems apparent from Table I that groupings 3 and 4 did not improve the error of the intersection, and values from grouping 2 are most reliable. This grouping, it may be noted, did not rely heavily upon the data obtained in the cretin.

These values for total serum thyroxine concentration (.0947 and 0.241 micromole per liter), correspond to .0736 and 0.187 microgram of thyroxine per ml., and to 4.82 and 12.3 micrograms of iodine per 100 ml. This is to be compared with the normal serum protein-bound iodine level of 4 to 8 micrograms per 100 ml.

In Figure 3, the concentration of thyroxine in albumin is plotted against total serum thyroxine on logarithmic coordinates. For those experiments in which more than 15 per cent of the total radioactivity was present in the gamma globulin area and at the origin of the paper strips (see above), an approximate correction for thyroxine displaced from albumin was provided by adding to the observed radioiodine in albumin that amount in gamma globulin plus origin which was in excess of 10 per cent. A total of 12 points in Figure 3 were corrected in this manner, and 8 of these were at total serum thyroxine levels greater than 1.0 micromole per liter. There again

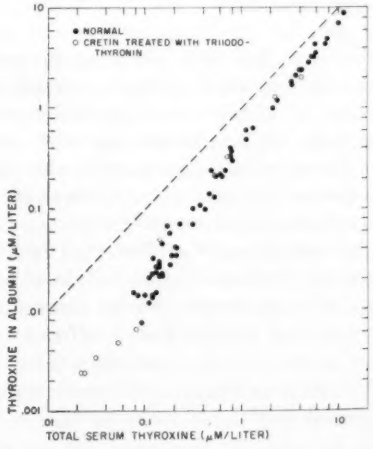


FIG. 3. THE CONCENTRATION OF ALBUMIN-BOUND THYROXINE AT INCREASING CONCENTRATIONS OF TOTAL SERUM THYROXINE

● = normals. ○ = cretin treated with triiodothyronine. The broken line represents 100 per cent binding of serum thyroxine to albumin.

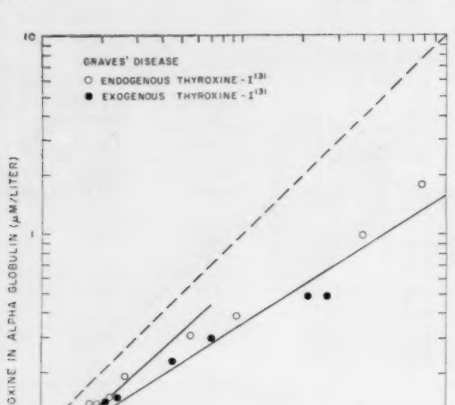


FIG. 4. A COMPARISON OF ENDOGENOUSLY LABELED AND EXOGENOUSLY LABELED THYROXINE IN THE SAME SERUM

○ = endogenously labeled thyroxine. ● = exogenously labeled thyroxine. The lines are identical with those in Figure 2. The serum was obtained 48 hours after radioiodine therapy in a patient with Graves' disease. See text for details.

appear to be two components defined by these data, but the slopes differ so little that it is impossible to be sure of this and no statistical treatment was attempted.

The data for thyroxine in beta and gamma globulins have not been subjected to analysis, inasmuch as they are likely to be in considerable error due to adsorption of albumin-thyroxine, and possibly alpha globulin-thyroxine, on the paper in these areas. Experiments with purified bovine gamma globulin (11, 12) have indicated little or no binding of thyroxine.

In the calculations described thus far, it has been implied that equilibrium had been reached between the endogenous serum thyroxine and added thyroxine, and that there was free exchange between these. These assumptions were tested on the serum from a patient with hyperthyroidism. This patient had classical Graves' disease, complicated by auricular fibrillation, and her disease was under partial control with propylthiouracil. (The serum thyroxine level was 0.14 microgram per ml.) Serum was obtained 48 hours after the administration of a therapeutic dose of radioiodide. Chromatographic analysis of the serum I^{131} (see above) indicated that 82 per cent of it was in the form of thyroxine. Five per cent behaved like iodide, 3 per cent like triiodothyronine, and 10 per cent remained at the origin, and may have been thyroglobulin (13). The thyroxine content of this serum was altered by the addition of unlabeled synthetic L-thyroxine, otherwise following the procedure for the normal sera. A recovery of 100 per cent of the added thyroxine was assumed for the calculation of total thyroxine content.

A second portion of the same serum was stored until the endogenous radioiodine had decayed to negligible proportions. Thyroxine-I^{131} was then added in the same way as with the normal sera.

The results of these experiments are presented in Figure 4. It can be seen that the data are similar, whether the radioactive label was on the endogenous thyroxine or on the added thyroxine. (In the case of the two highest points obtained with exogenously labeled thyroxine the recovery of added radioiodine was 40 and 66 per cent.) These results appear to justify the assumptions of equilibrium and free exchange between endogenous and added thyroxine, and, in addition, indicate that natural and synthetic thyroxine behave similarly under the conditions employed. It is uncertain whether the values obtained with this single hyperthyroid serum differ significantly from those obtained with normals.

DISCUSSION

It is apparent from the data in Figure 1 that the specific thyroxine-binding protein (TBP)[9] has a limited capacity to combine with excess quantities of thyroxine added to serum. Similar findings have been reported by Albright, Larson, and Deiss (14), and by Horst (15). Evaluation of the thyroxine-binding capacity of this protein, however, is beset with certain difficulties. In contrast to iron-binding phenomena in serum (16), for example, when the TBP has become saturated, binding of thyroxine to other serum proteins, notably to albumin, occurs. With the techniques

[9] The possibility exists that the specific thyroxine-binding protein referred to throughout this paper may in fact be a mixture of proteins. If such were the case, the term "thyroxine-binding protein" would more properly be described as the "thyroxine-binding sites" which are important at low serum thyroxine concentration.

employed in the present study, it has not been possible to isolate TBP from the other alpha globulins. In addition, it has been shown (17) that albumin (and thus any thyroxine which is bound to albumin), is adsorbed to the paper in the path of albumin migration.

It will be evident that the binding between thyroxine and TBP could not be evaluated in the usual manner (18), as has been done in the case of thyroxine and bovine serum albumin by Lein (19). In the present experiments, neither the concentration of TBP nor the concentration of unbound thyroxine were known, and purified TBP has not been available.

The use of a log-log function to describe the relationship of total serum thyroxine to thyroxine in alpha globulin was employed because it was the simplest function which fit the experimental data. This treatment lent itself to statistical analysis, and thus was of value, in the accompanying paper (20), as a basis for comparing the effects of various thyroxine analogues on thyroxine-binding in serum. A log-log relationship as a means of describing protein binding, although sometimes used, is of questionable physical significance with regard to the present data, and at very high or very low concentration of thyroxine, patently fallacious.

The occurrence of a shift in the proportions of thyroxine in the alpha globulin and albumin areas might be taken as an indication of saturation, or of beginning saturation, of the TBP. This takes place at a serum thyroxine level approximately 2 to 3 times normal, at which point the value for thyroxine in alpha globulin is 60 to 80 per cent of the total serum thyroxine. The corresponding values for the intersection of the two components in Figure 2 are considerably lower, but their significance with respect to the thyroxine-binding capacity of TBP are problematical. The values by both criteria are lower than the thyroxine-binding capacity of TBP estimated by Albright, Larson, and Deiss (14) for normal serum. While their value of 0.4 to 0.5 microgram per ml. for thyroxine in alpha globulin at saturation was based upon different criteria, it is to be noted that they assumed, probably incorrectly, that all of the endogenous thyroxine remained fixed to TBP during the analyses. When their data are recalculated, assuming equilibrium between endogenous and exogenous thyroxine, and an endogenous thyroxine level of 0.1 microgram per ml., the normal thyroxine-binding capacity of alpha globulin is approximately 0.3 microgram per ml., and is closer to the value indicated by the present study.

In contrast to the findings of Albright, Larson, and Deiss (14), the concentration of thyroxine in alpha globulin in the present study did not reach a plateau, but continued to increase throughout the range of thyroxine levels examined. This increase might be due to the binding of thyroxine to secondary sites on the alpha globulins, possibly on TBP itself. It might also be due to albumin-bound thyroxine which becomes fixed to the paper in the alpha globulin area. Preliminary experiments (21) have indicated that the latter alternative plays an important part in this phenomenon. In this regard, it may be of significance that Albright, Larson, and Deiss employed a different type of filter paper than that used in the present experiments.

The results of the present experiments can be considered valid only for the particular techniques used. It has not been established whether these results may differ when the binding phenomena are observed at different pH, in other buffers, or by techniques other than zone electrophoresis.

SUMMARY

The thyroxine-binding capacity of the specific thyroxine-binding protein (TBP) in normal serum has been studied by the use of thyroxine labeled with I^{131}, and zone electrophoresis at pH 8.6. It has been shown that this protein has a limited capacity to bind thyroxine, but the measurement of the actual binding capacity was complicated by the fact that excess thyroxine is bound to other serum proteins, notably to albumin, and the fact that the TBP could not be isolated from the other alpha globulins. It appears likely, however, that saturation of TBP occurs at serum thyroxine levels at least 2 to 3 times normal.

ACKNOWLEDGMENT

The authors wish to express their thanks to Dr. Rulon W. Rawson for his most helpful interest throughout this work, to Miss Helen Hagopian for excellent technical assistance, and to Miss Jane Heslin for help in the statistical analyses.

REFERENCES

1. Gordon, A. H., Gross, J., O'Connor, D., and Pitt-Rivers, R., Nature of the circulating thyroid hormone-plasma protein complex. Nature, 1952, **169**, 19.
2. Winzler, R. J., and Notrica, S. R., Association of thyroxine with plasma proteins. Federation Proc., 1952, **11**, 312.
3. Deiss, W. P., Albright, E. C., and Larson, F. C., A study of the nature of the circulating thyroid hormone in euthyroid and hyperthyroid subjects by use of paper electrophoresis. J. Clin. Invest., 1952, **31**, 1000.
4. Robbins, J., and Rall, J. E., Zone electrophoresis in filter paper of serum I^{131} after radioiodide administration. Proc. Soc. Exper. Biol. & Med., 1952, **81**, 530.
5. Horst, W., and Rösler, H., Der Transport des Hormonjods in menschlichen Serum untersucht mit Papier-elektrophorese und Radiojod. Klin. Wchnschr., 1953, **31**, 13.
6. Robbins, J., Petermann, M. L., and Rall, J. E., Electrophoresis of the thyroxine-binding protein of serum at pH 4.5. J. Biol. Chem., 1955, **212**, 403.
7. Petermann, M. L., Robbins, J., and Hamilton, M. G., Sedimentation of the thyroxine-binding protein of serum in the partition cell. J. Biol. Chem., 1954, **208**, 369.
8. Barker, S. B., Determination of protein-bound iodine. J. Biol. Chem., 1948, **173**, 715.
9. Gross, J., Leblond, C. P., Franklin, A. E., and Quastel, J. H., Presence of iodinated amino acids in unhydrolyzed thyroid and plasma. Science, 1950, **111**, 605.
10. Fisher, R. A., Statistical Methods for Research Workers. 11th Edition, Edinburgh, Oliver and Boyd, 1950, p. 144.
11. Taurog, A., and Chaikoff, I. L., The nature of the circulating thyroid hormone. J. Biol. Chem., 1948, **176**, 639.
12. Lein, A., The binding of thyroxin, diiodotyrosine and triiodothyronine by bovine serum proteins. Abstracts of papers, J. Am. Chem. Soc., March 15–19, 1953, p. 16 c.
13. Robbins, J., Thyroglobulin in serum after I^{131} therapy. I. Salting out. J. Biol. Chem., 1954, **208**, 377.
14. Albright, E. C., Larson, F. C., and Deiss, W. P., Thyroxine binding capacity of serum alpha globulin in hypothyroid, euthyroid, and hyperthyroid subjects. J. Clin. Invest., 1955, **34**, 44.
15. Horst, W., Neue Ergebnisse der Anwendung von J^{131} in Diagnostik und Therapie von Schilddrüsenerkrankungen. Strahlentherapie, 1954, **94**, 169.
16. Horst, W., and Schäfer, K. H., Die Eisenbindung im Serum und in weiteren biologischen Flüssigkeiten, untersucht mit Papierelektrophorese und Radioeisen (Fe^{59} und Fe^{55}). Zugleich eine Neue Methode zur Beurteilung der Eisenbindungskapazität Biologischer Flüssigkeiten. Klin. Wchnschr., 1953, **31**, 791.
17. Slater, R. J., and Kunkel, H. G., Filter paper electrophoresis with special reference to urinary proteins. J. Lab. & Clin. Med., 1953, **41**, 619.
18. Klotz, I. M., Protein interactions *in* Neurath, H., and Bailey, K., The Proteins. New York, Academic Press, Inc., 1953, vol. 1 B, p. 727.
19. Lein, A., Thyroxine binding by bovine serum albumin. Federation Proc., 1952, **11**, 91.
20. Robbins, J., and Rall, J. E., Effects of triiodothyronine and other thyroxine analogues on thyroxine-binding in human serum. J. Clin. Invest., 1955, **34**, 1331.
21. Robbins, J., Unpublished data.

EFFECTS OF TRIIODOTHYRONINE AND OTHER THYROXINE ANALOGUES ON THYROXINE-BINDING IN HUMAN SERUM [1]

By JACOB ROBBINS [2] AND J. E. RALL [3]

(From the Sloan Kettering Institute for Cancer Research, Memorial Center for Cancer and Allied Diseases, New York, N. Y.)

(Submitted for publication February 18, 1955; accepted April 20, 1955)

The strong affinity between thyroxine and the specific thyroxine-binding protein (TBP) of human serum (1) is apparent from, a) the inability to remove more than small amounts of thyroxine from the serum by prolonged dialysis (2), and b) the effectiveness with which TBP competes with albumin for available thyroxine in serum, despite its much smaller concentration. Thyroxine bound to TBP is, nevertheless, free to exchange with thyroxine added to serum *in vitro* (3). Thus, when endogenous serum thyroxine has been labeled with I^{131}, the addition of excess quantities of unlabeled thyroxine to such serum will cause a displacement of thyroxine-I^{131} from TBP. It may be expected that the addition to such serum of any substance capable of competing with thyroxine for its binding sites on the specific protein, would cause a similar displacement of thyroxine-I^{131}.

Earlier reports (4–6) have revealed that triiodothyronine has the ability to displace thyroxine from TBP. The present study represents an attempt to quantitate this effect, and to compare, in this way, the affinity of a series of thyroxine analogues for TBP.

MATERIALS AND METHODS

Sera from three healthy young adults (one female, two males) were obtained after overnight fasts, and were stored in the frozen state until used. The 14 analogues of thyroxine which were studied are listed in Table I, together with their sources of supply.[4] Each compound was dissolved in *n*-butanol at a concentration of 20 or 40 micrograms per ml. In the case of diiodothyronine and triiodothyronine, solution was favored by the addition of 0.3 per cent by volume of 2 N ammonium hydroxide. Three of the compounds (O-p-nitrobenzyl-L-tyrosine, and the 3',5'-dinitro and 3',5',3,5-tetranitro analogues of thyroxine) were incompletely dissolved, but a fine, and apparently homogeneous, suspension was obtained prior to the removal of aliquots.

Three batches of L-thyroxine, labeled with I^{131}, and dissolved in *n*-butanol were used.[5] Chromatographic analyses of these preparations (3) revealed that thyroxine-I^{131} comprised from 72 to 86 per cent (mean = 80 per cent) of the total radioactivity. From 3 to 5 per cent behaved like iodide, and from 10 to 25 per cent was due to contaminants of unknown nature.

Appropriate quantities of the L-thyroxine-I^{131} solution and each of the thyroxine analogue solutions were combined so as to produce fixed concentrations of thyroxine in the presence of varying concentrations of analogue. These mixtures were evaporated to dryness *in vacuo* without heating. Serum was then added, allowed to stand for 15 to 20 hours at 4° C., and then stored in the frozen state until used.

The concentration of thyroxine in the serum mixtures was determined by iodine analysis as previously described (3). Similar analysis was made for those analogues which contained iodine. In the case of the remaining analogues, the measured concentration (weight plus dilution factors) was used. Recovery of thyroxine-I^{131} after drying was assessed by radioactivity measurements as previously described (3), and it was assumed that identical recoveries pertained to the thyroxine analogues in each instance. These recoveries ranged from 68 to 115 per cent, but were usually between 85 and 100 per cent (mean = 93 per cent). The concentrations of thyroxine and analogues employed are indicated in Table I.

Each serum mixture was subjected to zone electrophoresis in filter paper at pH 8.6 (barbital buffer, ionic strength 0.1), and the distribution of radioiodine among the various serum protein components were determined. The techniques employed have been described elsewhere (3).

[1] This work was supported by a grant from the Damon Runyon Memorial Fund for Cancer Research, a grant from the American Cancer Society, and a contract with the Atomic Energy Commission, No. AT(30–1)–910.

[2] Present address: National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland.

[3] Alfred P. Sloan Foundation Scholar.

[4] The authors are indebted to these sources for their generosity in making the compounds available for this study.

[5] L-thyroxine was obtained through the courtesy of Smith, Kline and French Laboratories, Philadelphia, Pa., and was labeled with I^{131} by Abbott Laboratories, Oak Ridge, Tenn.

TABLE I

Binding of thyroxine analogues to TBP

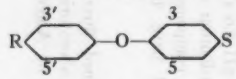
Compound								Analogue (μM/L.)	T_4 (actual)* (μM/L.)	No. of mixtures tested	Relative binding intensity† (%)
No.	R	3′	5′	3	5	S	Configuration				
Group A 											
1‡	HO	I	I	I	I	$CH_2CHCOOH$ | NH_2	L	—	—	—	100
2‡	HO	I	I	I	I	$CH_2CHCOOH$ | NH_2	D	.018 to 8.9	.14	7	80
3a‡	HO	H	I	I	I	$CH_2CHCOOH$ | NH_2	L	.043 to 41	.064 to .94**	25	35
3b§	HO	H	I	I	I	$CH_2CHCOOH$ | NH_2	L	.081 to 6.4	.13 to .17	8	31
4‖	HO	H	H	I	I	$CH_2CHCOOH$ | NH_2	L	3.5 to 30	.11 to .13	2	0
5‖	HO	CH_3	CH_3	I	I	$CH_2CHCOOH$ | NH_2	DL	.55 to 22	.14	4	4
6‖	HO	NO_2	NO_2	I	I	$CH_2CHCOOH$ | NH_2	L	.050 to 25	.14	6	47
7‖	HO	NO_2	NO_2	NO_2	NO_2	$CH_2CHCOOH$ | NH_2	DL	1.1 to 58	.14	4	1
8‖	AcO	I	I	I	I	$CH_2CHCOCH_3$ | NHAc		.053 to 23	.14	6	34
9¶	HO	H	I	I	I	CH_2COOH		1.2 to 58	.14	4	1
10‖	HO	H	H	I	I	$CH{=}CHCOOH$		63	.14	1	0
11‖	H	NO_2	NO_2	NO_2	NO_2	$CH_2CHCOOE_t$ | NHAc	DL	59	.14	1	0
12‖	$C_6H_5CH_2O$	CH_3	CH_3	NO_2	NO_2	$CH_2CHCOOE_t$ | NHAc	DL	61	.14	1	0

TABLE I—*Continued*

Compound No.	R	3'	5'	3	5	S	Configuration	Analogue ($\mu M/L$)	T_4 (actual)* ($\mu M/L$)	No. of mixtures tested	Relative binding intensity† (%)
Group B											
13¶	HO			I	I	$CH_2CHCOOH$ $\lvert$ NH_2	L	4.3 to 36	.11 to .13	2	0
14‖	HO			I	I	$COOCH_2CH_2CH_2CH_3$		60	.14	1	0
15‖	$p\text{-}NO_2C_6H_4CH_2O$			H	H	$CH_2CHCOOH$ $\lvert$ NH_2	L	38	.14	1	0

* Exogenous plus endogenous thyroxine.
† Relative to L-thyroxine. See text.
‡ Supplied by Dr. A. Heming, Smith, Kline, and French Laboratories, Philadelphia, Pa.
§ Supplied by Dr. J. Gross, New York, N. Y. (This compound was highly purified by column chromatography.)
¶ Supplied by Dr. B. Hems, Glaxo Laboratories, Middlesex, England.
‖ Supplied by Dr. R. Pitt-Rivers, Mill Hill, England.
** Less than .17 micromole per liter in 17 mixtures.

RESULTS

Electrophoretic distribution of radioiodine in serum containing a constant, small amount of L-thyroxine-I^{131} and increasing concentrations of L-triiodothyronine is illustrated in Figure 1. At high triiodothyronine concentrations, thyroxine-I^{131} is displaced from the alpha globulin area, and becomes associated with the other serum protein components, particularly with albumin. These results are analogous to those obtained when excess quantities of L-thyroxine are added to serum (3), but the molar concentration of L-triiodothyronine required to produce a given displacement is greater than in the case of thyroxine.

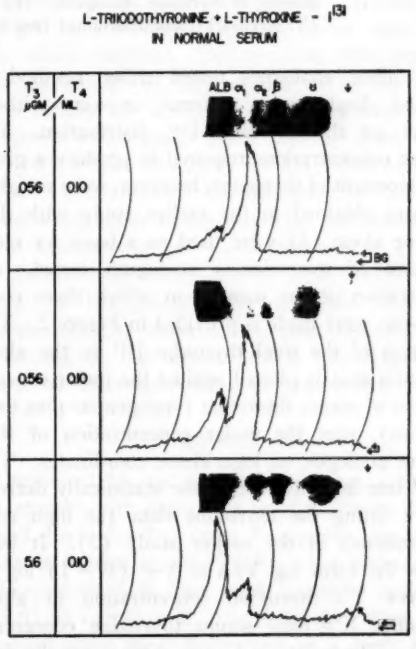


FIG. 1. THE EFFECT OF INCREASING CONCENTRATIONS OF L-TRIIODOTHYRONINE ON THE ELECTROPHORETIC DISTRIBUTION OF L-THYROXINE-I^{131} IN SERUM

T_3 represents added L-triiodothyronine, T_4 represents total serum L-thyroxine. In each instance, a continuous recording of the radioactivity on the paper strip (lower portion) is compared with the stained protein components on the same paper strip (upper portion). The single arrow denotes the point of application of serum to the paper; the double arrow, labeled "BG," represents background radioactivity. (Buffer: sodium barbital, pH 8.6, ionic strength 0.1.)

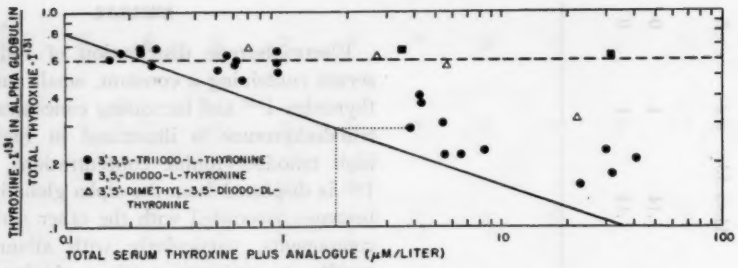


FIG. 2. DISPLACEMENT OF L-THYROXINE FROM SERUM ALPHA GLOBULIN BY THYROXINE ANALOGUES

The solid line was statistically derived from data obtained with L-thyroxine alone (3). The points represent thyroxine-analogue mixtures in which the total thyroxine concentration was between .06 and .17 micromole per liter. The dashed line indicates the lower limit of thyroxine in alpha globulin at normal thyroxine levels and in the absence of thyroxine analogues. The dotted line indicates the manner in which [T_4 (Theoretical)] was determined (see text).

The other analogues tested either resulted in similar displacement patterns, or were without effect on the thyroxine-I^{131} distribution. The molar concentrations required to produce a given displacement of thyroxine, however, were variable.

Data obtained in the earlier study with thyroxine alone (3) were used as a basis for comparison of the various analogues tested. An illustration of the manner in which these comparisons were made is provided in Figure 2. The fraction of the total thyroxine-I^{131} in the alpha globulin area is plotted against the molar concentration of serum thyroxine (endogenous plus exogenous), plus the molar concentration of thyroxine analogue, on logarithmic coordinates. The solid line is equivalent to the statistically derived curve fitting the thyroxine data (at high concentrations) in the earlier study (3). It here takes the form $\log Y/X = A + (B - 1) \log X$ (where Y = thyroxine concentration in alpha globulin, X = total serum thyroxine concentration). The points in Figure 2 represent the data obtained with three thyroxine analogues. It is apparent that L-triiodothyronine is less effective than L-thyroxine itself in producing displacement of thyroxine-I^{131} from alpha globulin, while L-diiodothyronine is without effect at the concentrations employed. The 3',5'-dimethyl analogue of DL-thyroxine exhibits an intermediate effect.

The dotted line in Figure 2 indicates the method of interpolation which provided an estimate of the total concentration of L-thyroxine, [T_4 (theoretical)], required to produce a degree of displacement equal to that actually produced by the analogue. The ability of the analogue to produce displacement was then compared to L-thyroxine by the expression:

$$\frac{[T_4\text{ (theoretical)}] - [T_4\text{ (actual)}]}{[\text{analogue}]} \times 100,$$

where the brackets indicate molar concentration. This calculation was made for each of the serum mixtures which fulfilled two criteria: 1) [analogue] was greater than [T_4 (actual)] by at least two-fold, and 2) the fraction of thyroxine-I^{131} in alpha globulin was less than 0.6. The averages of the calculated values for each analogue are recorded in Table I. Inasmuch as the ability of an analogue to cause displacement of L-thyroxine is in all likelihood due to competitive binding of the analogue itself to TBP, these values are listed under the heading, "Relative Binding Intensity." [6]

DISCUSSION

Certain considerations must be taken into account in evaluating these calculated values for relative binding intensities. First, the variations obtained in individual measurements with the same analogue are considerable. This is indi-

[6] References to the affinity of thyroxine analogues for TBP throughout this paper refer only to binding at the thyroxine-binding sites on this protein.

cated by the fact that the relative binding intensity for individual measurements with L-thyroxine itself ranged from approximately 50 to 150. (The scatter of the individual points around the straight line is shown in the previous study [3].) Second, the apparent affinity of a compound for TBP may be affected by any difference between it and L-thyroxine with regard to binding by other serum proteins. For example, a compound which is bound *only* by TBP would be likely to have a higher concentration of unbound compound than would thyroxine at comparable serum levels. Since binding to protein is proportional to the concentration of the unbound compound rather than to the total concentration, the value for "relative binding intensity" would be falsely high. Information bearing on this question is available only for triiodothyronine (4, 7), and it appears that triiodothyronine may be bound less strongly to albumin than is thyroxine. Thus, the values recorded in the table must be considered as approximations.

Nevertheless, the data permit certain tentative conclusions. The affinity of D-thyroxine (Table I, No. 2) for TBP does not appear to differ significantly from that of L-thyroxine (Table I, No. 1). None of the other analogues tested has a binding intensity as great as that of L-thyroxine itself, but a number of them demonstrate a considerable affinity for TBP. When those analogues with changes only in the 3′,5′,3,5 positions are considered (Table I, Nos. 3–7), it appears that one or more substituents in the 3′,5′ positions are essential for binding (*cf.* diiodothyronine—Table I, No. 4). This substituent, however, need not be iodine. Comparison of the 3′,5′-dimethyl and the 3′,5′-dinitro analogues suggest that substitution with the polar nitro groups produces stronger binding and, indeed, the tetranitro-analogue of thyroxine exhibits minimal binding activity. The different configurations of these compounds are unlikely to be a factor in view of the similarity between D- and L-thyroxine.

Replacement of the phenylalanine side chain of L-triiodothyronine with acetic acid (Table I, No. 9) does not entirely destroy binding, but weakens it greatly. Somewhat surprising is the result obtained with compound No. 8, which appears to indicate that alteration of all of the end groups of thyroxine still permits a considerable degree of binding to TBP. In the remaining analogues of Group A, the alterations in the molecule are too complex to permit interpretation at the present time.

Neither L-diiodotyrosine nor its analogues (Table I, Group B) are capable of displacing L-thyroxine from TBP at the concentrations tested.

While the role of TBP in thyroid physiology has not been established, it has been suggested that the difference between thyroxine and triiodothyronine with respect to binding to this protein may account for at least part of the observed differences in their *in vivo* effects (4). One of these *in vivo* differences is the much faster disappearance of intravenously administered triiodothyronine from the circulation (8–10). Similarly, it might be considered that the great rapidity with which injected diiodotyrosine disappears from the blood (11, 12) is due to its low or absent affinity for TBP. D-thyroxine, however, disappears with equally great rapidity from the blood (8), despite an affinity for TBP comparable to that of L-thyroxine. Consideration of D-thyroxine, which binds strongly to TBP but has little or no L-thyroxine-like metabolic effects (13) and L-triiodothyronine, which binds relatively weakly but has potent L-thyroxine-like effects, suggests that the molecular features responsible for binding to the serum protein are different from those concerned with metabolic activity.[7]

SUMMARY

A series of 14 analogues of L-thyroxine have been studied with regard to their ability to displace L-thyroxine-I^{131} from its specific binding protein in human serum (TBP). The technique of electrophoresis in filter paper (barbital buffer, pH 8.6) was employed. Earlier studies with L-thyroxine alone have been used as a basis for comparison, and the "relative binding intensities" of the analogues for the thyroxine-binding sites on TBP have been calculated.

Of the compounds tested, only D-thyroxine has a binding intensity as great as L-thyroxine.

[7] The D-thyroxine used in this study is from the same lot that had been tested earlier for turnover in the blood (8). It had also been checked for L-thyroxine-like effect by the goiter prevention assay, and had approximately 10 per cent of the activity of an equimolar dose of L-thyroxine (14).

Lesser degrees of binding are exhibited by analogues with changes in the 3',5' positions, such as L-triiodothyronine and the dinitro and dimethyl analogues of thyroxine. Binding is also retained by certain thyroxine analogues with changes in the phenolic hydroxyl, amino, and carboxyl groups. A number of analogues, including L-diiodothyronine and L-diiodotyrosine, fail to displace L-thyroxine from TBP in the concentrations employed.

The results obtained with certain of the compounds suggest that the molecular features of thyroxine responsible for binding to TBP are different from those concerned with metabolic activity.

ACKNOWLEDGMENTS

The authors wish to express their thanks to Dr. Rulon W. Rawson for his most helpful interest throughout this work, and to Miss Helen Hagopian for excellent technical assistance.

REFERENCES

1. Gordon, A. H., Gross, J., O'Connor, D., and Pitt-Rivers, R., Nature of the circulating thyroid hormone-plasma protein complex. Nature, 1952, **169**, 19.
2. Silver, S., The nature of the blood iodine and its determination. J. Lab. & Clin. Med., 1942, **28**, 329.
3. Robbins, J., and Rall, J. E., Thyroxine-binding capacity of serum in normal man. J. Clin. Invest., 1955, **34**, 1324.
4. Deiss, W. P., Albright, E. C., and Larson, F. C., Comparison of *in vitro* serum protein binding of thyroxin and triiodothyronine. Proc. Soc. Exper. Biol. & Med., 1953, **84**, 513.
5. Robbins, J., and Rall, J. E., Studies on the thyroxine-protein complex of serum. J. Clin. Endocrinol. & Metab., 1954, **14**, 772.
6. Horst, W., Transport und Bindung im Serum. Untersucht mit Papierelektrophorese und Radioaktiven Indicatoren (Fe 55/59, Cu 64, Co 56/57, Mn 52, Ga 67, S 35, I 131). Klin. Wchnschr., 1954, **32**, 961.
7. Robbins, J., and Rall, J. E., Unpublished data.
8. Rall, J. E., Robbins, J., Becker, D., and Rawson, R. W., The metabolism of labeled L-triiodothyronine, L-thyroxine and D-thyroxine. J. Clin. Invest., 1953, **32**, 596.
9. Rawson, R. W., Rall, J. E., Pearson, O. H., Robbins, J., Poppell, H. F., and West, C. D., L-triiodothyronine versus L-thyroxine. A comparison of their metabolic effects in human myxedema. Am. J. M. Sc., 1953, **226**, 405.
10. Sterling, K., Lashof, J. C., and Man, E. B., Disappearance from serum of I^{131}-labeled L-thyroxine and L-triiodothyronine in euthyroid subjects. J. Clin. Invest., 1954, **33**, 1031.
11. Albert, A., and Keating, F. R., Metabolic studies with I^{131} labeled thyroid compounds: Distribution and excretion of radiodiiodotyrosine in human beings. J. Clin. Endocrinol., 1951, **11**, 996.
12. Tong, W., Taurog, A., and Chaikoff, I. L., The Metabolism of I^{131}-labeled diiodotyrosine. J. Biol. Chem., 1954, **207**, 59.
13. Reineke, E. P., and Turner, C. W., The relative thyroidal potency of l- and d,l-thyroxine. Endocrinology, 1945, **36**, 200.
14. Money, W. L., and Rawson, R. W., Unpublished data.

PARTIAL CHARACTERIZATION OF URINARY ADRENOCORTICAL STEROIDS IN ADRENAL HYPERPLASIA [1]

By WALTER R. EBERLEIN and ALFRED M. BONGIOVANNI

(From the Department of Pediatrics, University of Pennsylvania, and the Endocrine Division, Children's Hospital of Philadelphia, Philadelphia, Pa.)

(Submitted for publication December 13, 1954; accepted April 6, 1955)

Following the demonstration by Wilkins, Lewis, Klein, and Rosenberg (1) that cortisone controls the clinical manifestations and abnormal laboratory findings in virilizing adrenal hyperplasia, Bartter, Albright, Forbes, Leaf, Dempsey, and Carroll (2) postulated a deficient synthesis of "sugar hormone" (17-hydroxycorticosterone, Compound F) in this disease, reasoning from the unusual response of such patients to the administration of adrenocorticotropin (ACTH). This hypothesis has been supported by the demonstration of low blood levels of 17-hydroxylated-corticosteroids ("Porter-Silber chromogens") in untreated patients with adrenal hyperplasia and the usual failure of these levels to rise, following administration of ACTH (3,4). The discovery that, routinely, large quantities of pregnane-3α, 17α,20α-triol are excreted in the urine of patients with virilizing adrenal hyperplasia (5) has suggested a possible site of the metabolic block in the synthesis of Compound F.

The present studies were undertaken to characterize the corticosteroids excreted in the urine in adrenal hyperplasia, in an attempt to define more precisely the specific defect in steroid biosynthesis.

METHODS

Twenty-four-hour collections of urine from normal control subjects and from patients with adrenal hyperplasia were investigated in the following manner:

Urine for 17-ketosteroid determinations was hydrolyzed first with beta-glucuronidase [2] and manually extracted with ether; the remaining urine was continuously extracted at room temperature with ether for 36 hours after the addition of 10 volumes per cent (v/v) of 40 per cent sulfuric acid. The combined extracts were fractionated on alumina columns (6) and suitable fractions were submitted to infra-red spectrophotometric analysis.

The level of urinary neutral 17-ketosteroids produced by bismuthate oxidation ("17-ketogenic steroids") was determined according to the method of Brooks and Norymberski (7).

The quantities of pregnane-3α,17α,20α-triol were determined in urine hydrolyzed with beta glucuronidase [2] and chromatographed on alumina according to the method of one of the authors (5).

Crude extracts of urine hydrolyzed with beta glucuronidase were chromatographed on florisil (8). The fraction eluted with 25 per cent methanol in chloroform was examined by the following techniques: a) The phenylhydrazine-sulfuric acid method of Porter and Silber (9); b) periodate oxidation for the simultaneous determination of formaldehydogenic and acetaldehydogenic steroids (10); c) phosphomolybdate method of Heard and Sobel (11); d) blue tetrazolium method of Chen, Wheeler, and Tewell (12); e) 2,4-dinitrophenylhydrazine method of Gornall and Macdonald (13).

Urines collected from patients during therapy with cortisone were obtained two weeks to six months following the introduction of the intramuscular administration of Compound E acetate in doses ranging from 25 to 75 mg. given every third day. In each case, the specimen studied was obtained at a time when the total urinary 17-ketosteroids were significantly depressed (1).

Each of the methods described is designed to estimate the quantities of steroids with functional groups as follows. The determination of 17-ketosteroids formed by bismuthate oxidation measures only those compounds with 17,20-dihydroxy, 21 methyl groupings. The phenylhydrazine-sulfuric acid method which determines so-called "Porter-Silber chromogens," measures 17,21-dihydroxy-20-carbonyl side-chains only. Periodate oxidation of urine residues releases formaldehyde from those compounds with a 21-hydroxy group having either a 20-hydroxy or 20-carbonyl substituent, regardless of the presence or absence of a 17-hydroxyl function. Periodate oxidation will release acetaldehyde only from steroids with 17,20-dihydroxy, 21-methyl side-chains and hence has the same significance as the 17-ketogenic compounds indicated by bismuthate oxidation. Both the phosphomolybdate and blue tetrazolium techniques measure reducing substances or steroids with an alpha-ketolic side-chain, although the former will also react with molecules possessing an alpha-beta unsaturated system in ring A. The reagent, 2,4-dinitrophenylhydrazine, reacts with various carbonyl functions on the molecule, resulting in the

[1] This work was made possible by a research grant (A-619 R) from the Division of Research Grants and Fellowships, of the National Institutes of Health, United States Public Health Service.

[2] "Ketodase," Warner-Chilcott Co.

formation of complexes showing various maxima between 400 to 500 mμ, depending on the number and location of such groupings, as described by Gornall and Macdonald (13).

PAPER CHROMATOGRAPHY

Twenty-four-hour collections of urine from a normal control subject, a patient with Cushing's syndrome, and a patient with untreated adrenal hyperplasia were extracted at neutral pH, and again after glucuronidase hydrolysis, with chloroform. The separate washed extracts were fractionated on florisil columns, as above, and applied to paper. The solvent systems modified after Bush (14) were: Benzene: 50 per cent methanol (B5); iso-octane-toluene: 70 per cent methanol (E_1); and iso-octane: 90 per cent methanol (E_2). Chromatograms were equilibrated at 32° and developed at room temperature for 3 to 12 hours. The positions of Cpd. F, Cpd. E, pregnane-3α,11β,17α,21-tetrol-20-one (tetrahydro-F), and pregnane-3α,17α,21-triol-11, 20-dione (tetrahydro-E) were recognized by scanning chromatograms under ultraviolet light and staining central strips with a methanolic-sodium hydroxide solution of triphenyltetrazolium hydrochloride (TPZ), permitting simultaneous detection of reducing steroids, steroids with the dihydroxyacetone side chain (15), and others with the Δ4-3-keto configuration of Ring A (16). The eluted areas were dried, acetylated, and rechromatographed in the slower systems E_1 and E_2 against standards acetylated in the same manner. Final identification rested on these criteria: Mobility of the free compound and its acetate compared to standards on the same chromatogram; the reactions with TPZ and NaOH; the fluorescence on paper of Cpd. F treated with concentrated sulfuric acid (17); the blue color given by Cpd. E treated with iodine-potassium iodide (17); the fluorescent reactions of tetrahydro-F and tetrahydro-E treated with 15 per cent phosphoric acid (18); the spectra of the four acetates in sulfuric acid compared to those of pure compounds reported in the literature (17, 19).

RESULTS

Figure 1 illustrates a typical chromatographic study of the neutral 17-ketosteroids excreted by patients with adrenal hyperplasia, before and during treatment with cortisone. It is clear that not only is the total amount of 17-ketosteroids elevated prior to treatment but that the quantities of 11-oxygenated-17 ketosteroids, identified as 11-keto-etiocholanolone and 11-hydroxyandrosterone by infra-red analysis, are disproportionately increased. In this laboratory the 11-oxygenated compounds were found to constitute about 10 per cent of the total neutral 17-ketosteroids excreted by adult male and female controls and 18 to 25 per cent of the total in individuals with adrenal hyperplasia.

The results of bismuthate oxidation are indicated in Figure 2 for six patients with untreated adrenal hyperplasia, compared to nine normal controls. The 24-hour excretion of pregnane-3α,

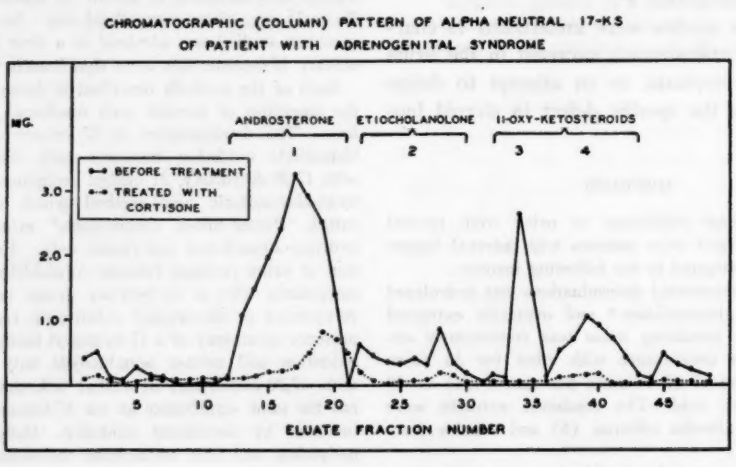


FIG. 1.

The ordinate indicates milligrams of 17-ketosteroids per fraction and the abscissa, the individual fractions in order of their elution from the column. The solid line illustrates the results before treatment and the dotted line during treatment with cortisone.

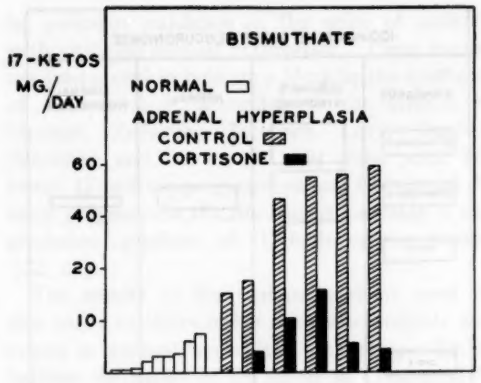


FIG. 2. TWENTY-FOUR-HOUR EXCRETION IN THE URINE OF 17-KETOSTEROIDS LIBERATED BY BISMUTHATE OXIDATION.

The first 4 "normal" columns and the first 2 "adrenal hyperplasia" columns represent children under the age of nine years. The black columns represent the excretion of these substances during the administration of cortisone.

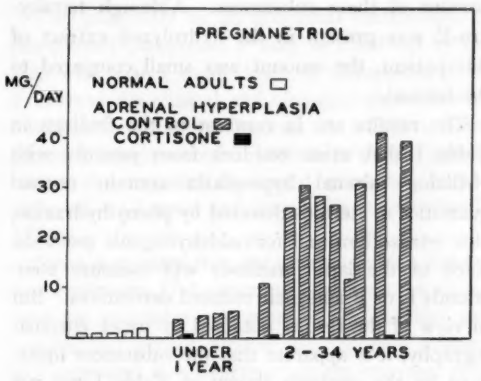


FIG. 3. TWENTY-FOUR-HOUR EXCRETION OF PREGNANE-3α,17α,20α-TRIOL IN FIVE NORMAL ADULTS, CONTRASTED WITH CHILDREN AND ADULTS WITH ADRENAL HYPERPLASIA

The suppressed excretion during cortisone treatment is indicated for two individuals.

17α,20α-triol is indicated in similar fashion in Figure 3.

Table I lists the results of the various assay methods applied to the steroid fractions obtained from florisil columns. The fractions employed were free of 17-ketosteroids but contained crystalline mixtures of several C–21 steroids. It is evident that the amount of acetaldehydogenic steroids excreted in adrenal hyperplasia is more than 10 times the amount found in the urine of normal controls. These patients also excrete greater than normal quantities of material detected by the phosphomolybdate reagent.

Figures 4 and 5 illustrate in condensed form the results of a series of 27 chromatograms run on extracts of unhydrolyzed and glucuronidase-hydrolyzed urine from a patient with adrenal hyperplasia, compared to a normal control subject and an individual with Cushing's syndrome. No Compound F or Compound E was detected in the unhydrolyzed extract of the patient with adrenal hyperplasia: 30 liters of urine from another patient so studied also contained no detectable

TABLE I

Twenty-four-hour excretion of corticosteroids in urine *

Corticosteroid assay method	Normal			Adrenal hyperplasia		
	No. subjects	Average *mg.*	Range *mg.*	No. subjects	Average *mg.*	Range *mg.*
Phenylhydrazine†	7	8.5	3.8–13.1	7	6.1	3.8–8.6
Phosphomolybdate†	7	13.0	5.5–24.4	7	29.7	19.8–44.0
Blue Tetrazolium†	4	6.5	2.9–9.0	4	7.7	5.0–10.6
2,4-Dinitrophenylhydrazine†	5	8.8	2.5–18.4	8	4.5	1.8–11.8
		mcg.	*mcg.*		*mcg.*	*mcg.*
Formaldehydogenic‡	8	374	210–671	8	371	235–618
Acetaldehydogenic‡	7	179	50–456	7	2,014	1,249–3,244

* Comparison is made of the urinary excretion by normal individuals and untreated patients with virilizing adrenal hyperplasia, of corticosteroids measured by the procedures described. These are to be contrasted with the findings obtained by paper chromatography (Figures 4 and 5) as described in the text.

† Calculated as milligrams of Cpd. E.

‡ Micrograms of formaldehyde or acetaldehyde released by periodate oxidation.

amount of these substances. Although tetrahydro-E was present in the hydrolyzed extract of this patient, the amount was small compared to the normal.

The results are in contrast to the findings in Table I that urine residues from patients with virilizing adrenal hyperplasia contain normal quantities of steroids detected by phenylhydrazine, blue tetrazolium and formaldehydogenic methods. Each of the latter methods will measure compounds E or F and their reduced derivatives. But in view of the results obtained by paper chromatography, it is apparent that the substances measured by the methods shown in Table I are not Compounds E or F or their reduced metabolites, as is the case in the urine of normal individuals. Nonetheless, unidentified 21-hydroxylated steroids are present in the urine of untreated patients with adrenal hyperplasia.

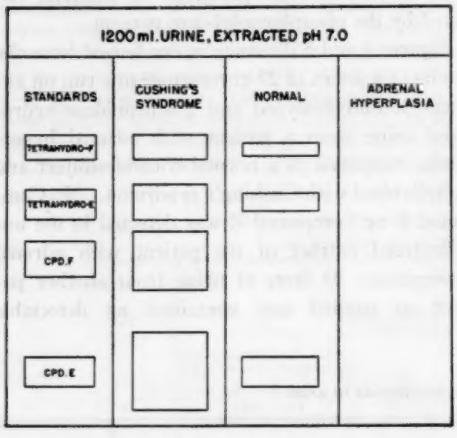


FIG. 4. CONDENSATION OF MULTIPLE PAPER CHROMATOGRAMS RUN ON EXTRACTS OF UNHYDROLYZED URINE FROM A NORMAL SUBJECT, A PATIENT WITH CUSHING'S SYNDROME, AND A PATIENT WITH VIRILIZING ADRENAL HYPERPLASIA TO ISOLATE COMPOUND F, COMPOUND E, AND THEIR TETRAHYDRO METABOLITES

The first column indicates the relative position on the chromatograms of the 4 steroids. The rectangles in columns 2 to 4 indicate the relative amount of the individual steroid excreted, estimated by spot size and staining intensity on the original chromatograms. Note the excess excretion of the 4 steroids in Cushing's syndrome and the complete absence of all in adrenal hyperplasia.

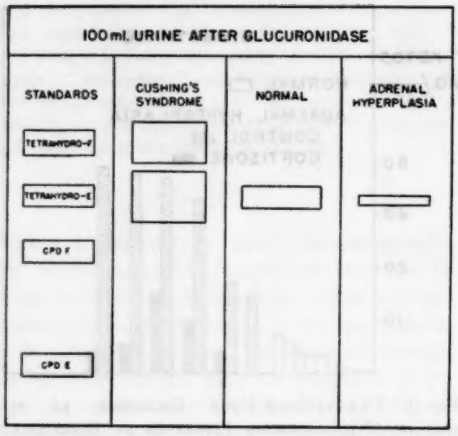


FIG. 5. CONDENSATION OF MULTIPLE CHROMATOGRAMS OF GLUCURONIDASE-HYDROLYZED URINE AFTER INITIAL EXTRACTION AT NEUTRAL pH

Patients and symbols as in Figure 4. Note the small excretion of tetrahydro-E in adrenal hyperplasia, the excessive amount of the same steroid in Cushing's syndrome.

DISCUSSION

The present investigation of the excretion of Compound F, Compound E, and their known end-metabolites, tetrahydro-F and tetrahydro-E, studied by means of paper chromatography, firmly supports the theory that there is a deficient synthesis of Compound F in adrenal hyperplasia. At the same time, and in agreement with previous studies on the response of the blood "Porter-Silber chromogen" levels to ACTH administration (4), the findings indicate that at least some of these patients can synthesize a small amount of Compound F, evidenced by the excretion of a small amount of tetrahydro-E derived from Compound F.

The most characteristic, and hence diagnostic, laboratory finding in virilizing adrenal hyperplasia remains the excretion of large amounts of pregnane-3α,17α,20α-triol in the urine. This steroid and its recently isolated relative, pregnane-3α,17α,20α-triol-11-one (20), appear to account for the excessive amounts of 17-ketosteroids liberated by bismuthate oxidation and for the large amounts of acetaldehydogenic steroids detected

by periodate oxidation in the urine of patients with virilizing adrenal hyperplasia. These results are interpreted to indicate a block in the synthesis of Compound F, according to the scheme of Hechter, Zaffaroni, Jacobsen, Levy, Jeanloz, Schenker, and Pincus (21), at some point between 17-hydroxyprogesterone and Compound F, since pregnane-3α,17α,20α-triol is probably a degradation product of 17-hydroxyprogesterone (22, 23).

The results of the various methods used in this study to characterize the corticosteroids excreted in adrenal hyperplasia do not specifically indicate the nature of the defect in Compound F synthesis. According to Hechter's scheme, based upon adrenal perfusion experiments, cholesterol, under the stimulus of ACTH, is converted *via* pregnenolone into progesterone, which is then successively hydroxylated at carbons 17, 21, and 11 to yield Compound F. It has been shown that adrenal tissue contains enzymes capable of introducing hydroxyl groups into suitable substrates at these three positions *in vitro* (24, 25), although none of these enzymes has as yet been isolated in a pure state. It has already been shown that endogenous ACTH production is high in virilizing adrenal hyperplasia (26), hence one cannot incriminate the absence of an adequate stimulus to Compound F production. Judging from the present study, there is no deficiency of the enzyme which regulates 17-hydroxylation. The excretion of normal quantities of steroids possessing an alpha-ketolic side chain, measured by formaldehyde liberation, as well as the blue tetrazolium and Porter-Silber methods, does not readily suggest a deficiency of a 21-hydroxylating enzyme. Nor does the presence in the urine of large amounts of 11-oxygenated-17-ketosteroids and of pregnanetriol-11-one point to the lack of an 11-hydroxylating enzyme.

Indeed, in crude extracts of urine from patients with adrenal hyperplasia one can detect normal or excessive amounts of steroids, which in the aggregate possess all the necessary molecular configurations of Compound F itself. Unidentified steroids unsaturated in Ring A are demonstrable on paper chromatograms and presumably account for the high levels detected with the phosphomolybdate reagent.

The possibility of an abnormal metabolism of pre-formed Compound F may be ruled out by the ease with which small doses of Compound F or E reverse the clinical and chemical abnormalities found in the untreated disease. In addition, Compound E administered to patients with the disease causes the expected increased excretion in the urine of its known end-metabolite, tetrahydro-E (27).

Since it appears that the adrenal in this disease can synthesize 17-hydroxyprogesterone, as measured by excessive pregnane-3α,17α,20α-triol excretion, but cannot produce an adequate amount of Compound F, a deficiency in the enzymes concerned with hydroxylation either at carbon 11 or at carbon 21 might be postulated. The present study offers several objections to the theory of "11β-hydroxylase" deficiency in adrenal hyperplasia. Since 11-hydroxylation follows 21-hydroxylation (21), one would not expect a block at this site to lead to the marked excretion of pregnane-3α,17α,20α-triol but rather of 21-hydroxylated steroids, such as 17-hydroxy-11-desoxycorticosterone and its reduced derivative, which should be measurable as alpha-ketolic steroids both in blood and urine. The latter compounds have not so far been detected in the urine or blood of patients with virilizing adrenal hyperplasia.

Another objection to "11β-hydroxylase" lack is the excess excretion of 11-oxygenated steroids which occurs. In respect to this, however, in adrenal perfusion experiments, but not in studies using adrenal homogenates, some 11-hydroxylation of 21-desoxy steroids, such as progesterone, can occur (21). This suggests that in the intact adrenal there may be normally two enzyme systems concerned with 11β-hydroxylation, one dependent upon, and the other independent of, prior hydroxylation at carbon 21. If this is true, the 11-oxygenated steroids excreted in adrenal hyperplasia might derive primarily from 21-desoxy precursors through the action of the latter enzyme.

Dorfman (28) has suggested a deficiency of a 21-hydroxylating enzyme in adrenal hyperplasia. The theory is compatible with the high excretion of pregnane-3α,17α,20α-triol in the urine and the low levels of Porter-Silber chromogens in the blood. The present study reveals that pa-

tients with this disease excrete approximately the same amount of alpha-ketolic (*i.e.*, 21-hydroxylated) steroids as do normal individuals, and that most of these steroids detected with the Porter-Silber reagent are hydroxylated at carbon 17, although some may have 16-hydroxyl groups (29). Since endogenous ACTH production is high, the amount of alpha-ketolic steroids excreted may be relatively low. But it is difficult to explain why, in the presence of more than adequate amounts of enzymes capable of inducing hydroxylation at carbon 11, the adrenal cortex is unable to synthesize more respectable quantities of Compound F from precursors already oxygenated at C–17 and C–21. In other words, the amount of Compound F and its metabolites constitutes a significantly smaller proportion of the total alpha-ketolic steroids excreted in the urine of subjects with this adrenal disorder than in normal controls.

The present study, therefore, does not support the theory of either a single "11β-hydroxylase" or "21-hydroxylase" deficiency in adrenal hyperplasia, reasoning from the current concepts of steroid biosynthesis in the adrenal cortex. The presumed inborn error of metabolism is apparently more complicated and may well involve a combination of enzymes and their cofactors. Work in progress in this laboratory, including final identification of the alpha-ketolic steroids excreted in the urine, may help to clarify the underlying disturbance of Compound F synthesis.

SUMMARY

A study, utilizing both paper and column chromatographic techniques, revealed an abnormally low excretion of Compound F and its metabolites in the urine of patients with adrenal hyperplasia. This strongly supports the concept of a deficient synthesis of Compound F by the diseased adrenal cortex. The characteristic excretion of pregnane-3α,17α,20α-triol in large quantities suggests a deficient conversion of 17-hydroxyprogesterone into Compound F. Large amounts of steroids oxygenated at C–11 and at C–17 and "normal" quantities of 21-hydroxylated steroids were detected in the urine of several affected individuals. The nature of the underlying enzymatic defect in the synthesis of Compound F in this disease awaits precise definition.

Addendum

A patient with congenital adrenal hyperplasia complicated by hypertension, since studied, excreted in the urine predominantly tetrahydro-S (pregnane-3α,17α,20-triol-20-one, confirmed by infra-red analysis), a finding hitherto unreported in this disease. Complete absence in the urine of the normal 11-oxygenated C-21 and 17-ketosteroids suggests "11β-hydroxylase" deficiency in this form of the disease. (Eberlein, W. R., and Bongiovanni, A. M., J. Clin. Endocrinol. & Metab., Submitted for publication.)

ACKNOWLEDGMENTS

The authors acknowledge with pleasure the helpful suggestions of Doctors Lawson Wilkins and Nathan Talbot. The infra-red analyses were performed through the kindness of Doctor Seymour Lieberman. Samples of pure steroids were generously supplied by Doctors John Schneider, Hirsch Sulkowitch, Harold Mason, The G. D. Searle Company, The Squibb Institute for Medical Research and Merck and Company.

REFERENCES

1. Wilkins, L., Lewis, R. A., Klein, R., and Rosenberg, E., The suppression of androgen secretion by cortisone in a case of congenital adrenal hyperplasia. Bull. Johns Hopkins Hosp., 1950, **86**, 249.
2. Bartter, F. C., Albright, F., Forbes, A. P., Leaf, A., Dempsey, E., and Carroll, E., The effects of adrenocorticotropic hormone and cortisone in the adrenogenital syndrome associated with congenital adrenal hyperplasia: An attempt to explain and correct its disordered hormonal pattern. J. Clin. Invest., 1951, **30**, 237.
3. Ely, R. S., Kelley, V. C., and Raile, R. B., Studies of 17-hydroxycorticosteroids in children. I. Peripheral blood levels in health and disease. J. Pediat., 1953, **42**, 38.
4. Bongiovanni, A. M., Eberlein, W. R., and Cara, J., Studies on the metabolism of adrenal steroids in the adrenogenital syndrome. J. Clin. Endocrinol. & Metab., 1954, **14**, 409.
5. Bongiovanni, A. M., and Clayton, G. W., Jr., A simplified method for the routine determination of pregnanediol and pregnanetriol in urine. Bull. Johns Hopkins Hosp., 1954, **94**, 180.
6. Dingemanse, E., Huis in't Veld, L. G., and Hartogh-Katz, S. L., Clinical method for the chromatographic-colorimetric determination of urinary 17-ketosteroids. II. Normal adults. J. Clin. Endocrinol. & Metab., 1952, **12**, 66.
7. Brooks, C. J. W., and Norbymberski, J. K., The oxidation of corticosteroids with sodium bismuthate. Biochem. J., 1953, **55**, 371.
8. Sandberg, A. A., Nelson, D. H., Glenn, E. M., Tyler, F. H., and Samuels, L. T., 17-hydroxycorticosteroids and 17-ketosteroids in urine of human sub-

jects: Clinical application of a method employing β-glucuronidase hydrolysis. J. Clin. Endocrinol. & Metab., 1953, **13**, 1445.

9. Porter, C. C., and Silber, R. H., A quantitative color reaction for cortisone and related 17,21-dihydroxy-20-ketosteroids. J. Biol. Chem., 1950, **185**, 201.
10. Cox, R. I., A method for the quantitative determination in urinary extracts of C_{21}–17:20-dihydroxy-21-methylsteroids. Biochem. J., 1952, **52**, 339.
11. Heard, R. D. H., and Sobel, H., A colorimetric method for the estimation of reducing steroids. J. Biol. Chem., 1946, **165**, 687.
12. Chen, C., Wheeler, J., and Tewell, H. E., Jr., Methods of estimation of adrenal cortical steroids with tetrazolium salts. J. Lab. & Clin. Med., 1953, **42**, 749.
13. Gornall, A. G., and Macdonald, M. P., Quantitative determination of the steroid hormones with 2,4-dinitrophenylhydrazine. J. Biol. Chem., 1953, **201**, 279.
14. Bush, I. E., Methods of paper chromatography of steroids applicable to the study of steroids in mammalian blood and tissues. Biochem. J., 1952, **50**, 370.
15. Axelrod, L. R., A specific test differentiating between α-ketol and dihydroxy-acetone groups of C_{21}-steroids on paper chromatograms. J. Am. Chem. Soc., 1953, **75**, 4074.
16. Bush, I. E., and Sandberg, A. A., Adrenocortical hormones in human plasma. J. Biol. Chem., 1953, **205**, 783.
17. Zaffaroni, A., and Burton, R. B., Identification of corticosteroids of beef adrenal extract by paper chromatography. J. Biol. Chem., 1951, **193**, 749.
18. Neher, R., and Wettstein, A., Über Steroide. Farbreaktionen mit Steroiden, insbesondere Corticosteroiden, im Papierchromatogramm. Helvet. chim. acta, 1951, **34**, 2278.
19. Bernstein, S., and Lenhard, R. H., The absorption spectra of steroids in concentrated sulfuric acid. I. Method and data. J. Org. Chem., 1953, **18**, 1146.
20. Finkelstein, M., Euw, J. V., and Reichstein, T., Isolierung von 3α,17,20α-trioxy-pregnanon-(11) aus pathologischem menschlichem Harn. Helvet. chim. acta, 1953, **36**, 1266.
21. Hechter, O., Zaffaroni, A., Jacobsen, R. P., Levy, H., Jeanloz, R. W., Schenker, V., and Pincus, G., The nature and the biogenesis of the adrenal secretory product. Rec. Prog. Hormone Res., 1951, **6**, 215.
22. Mason, H. L., and Kepler, E. J., Isolation of steroids from the urine of patients with adrenal cortical tumors and adrenal cortical hyperplasia: A new 17-ketosteroid, androstane-3α,11-diol-17-one. J. Biol. Chem., 1945, **161**, 235.
23. Bongiovanni, A. M., Unpublished data.
24. Hayano, M., Dorfman, R. I., and Yamada, E. Y., The conversion of desoxycorticosterone to glycogenic material by adrenal homogenates. J. Biol. Chem., 1951, **193**, 175.
25. Plager, J. E., and Samuels, L. T., Enzyme system involved in the oxidation of carbon-21 in steroids. Federation Proc., 1952, **11**, 383.
26. Sydnor, K. L., Kelley, V. C., Raile, R. B., Ely, R. S., and Sayers, G., Blood adrenocorticotrophin in children with congenital adrenal hyperplasia. Proc. Soc. Exper. Biol. & Med., 1953, **82**, 695.
27. Eberlein, W. R., Unpublished data.
28. Dorfman, R. I., The metabolism of adrenal steroids *in* Adrenal Cortex. Transactions of the Fifth Conference, 1953, New York, Josiah Macy, Jr. Foundation, 1954, p. 27.
29. Mattox, V. R., Mason, H. L., and Albert, A., Isolation of a sodium-retaining substance from beef adrenal extracts. Proc. Staff Meet., Mayo Clin., 1953, **28**, 569.

CLINICAL SCIENCE

Edited for the Medical Research Society by
W. MELVILLE ARNOTT

Assistant Editors
G. D. DAWSON, K. W. DONALD, H. E. DE WARDENER

Advisory Board

E. A. CARMICHAEL
R. V. CHRISTIE
R. T. GRANT
J. McMICHAEL
G. W. PICKERING
E. E. POCHIN
E. P. SHARPEY-SCHAFER
JANET VAUGHAN
W. C. WILSON
L. J. WITTS

Volume 14, No. 2. **Issued May 1955**

Further Studies on the Mechanism of Experimental Hypertension in the Rat. By M. A. FLOYER.

Relation Between Blood Sugar Level and the Optical Properties of the Lens of the Human Eye. By D. W. VERE and D. VEREL.

Reactive Hyperaemia in the Human Forearm. By G. C. PATTERSON and R. F. WHELAN.

Anatomical Closure of the Ductus Arteriosus in the Guinea Pig. By R. G. RECORD and T. McKEOWN.

The Effect of Reduced Atmospheric Pressure on Closure of the Ductus Arteriosus in the Guinea Pig. By R. G. RECORD and T. McKEOWN.

Diurnal Variation in the turnover of Iron through the Plasma. By T. H. BOTHWELL and BARBARA MALLETT.

Renal Uptake of Radioactive Potassium. By E. W. EMERY, R. HOLMES, H. E. F. DAVIES and D. A. K. BLACK.

Distribution of Labelled Gamma Globulin in Humans Studied with an External Counter. By N. B. MYANT.

"Fixation" of Tuberculin in the Skin of Tuberculin Sensitive Human Subjects. By J. PEPYS.

The Effect of Intra-Arterial Antihistamines on the Hyperaemia following Temporary Arrest of the Circulation in the Human Forearm. By F. DUFF, G. C. PATTERSON and R. F. WHELAN.

The Rate of Blood Flow and the Oxygen Saturation of the Effluent Blood Following Contraction of the Muscles of the Human Forearm. By A. H. G. LOVE.

The Effect of Venous Congestion on the Rate of Heat Elimination from the Fingers. By R. G. SHANKS.

The Urinary Excretion of Labelled Vitamin B12. By SHEILA T. CALLENDER and J. R. EVANS.

The Relationship Between Blood Flow, Capillary Surface Area and Sodium Clearance in Muscle. By D. N. WALDER.

The Role of Oxygen, Carbon Dioxide and Lactic Acid in the Ventilatory Response to Exercise in Patients with Mitral Stenosis. By J. E. COTES.

Circulatory Dynamics at Rest and on Exercise in the Hyperkinetic States. By J. M. BISHOP, K. W. DONALD and O. L. WADE.

Shaw & Sons Ltd., 7-9 Fetter Lane, Fleet Street, London, E. C. 4, England

Subscription Rate: £3, 15. 0d. per volume.

REAGENTS and REACTIONS

from dahlias and artichokes...

... comes a polysaccharide known as inulin. According to the N.N.R., "inulin clearance is the most accurate measure" of glomerular filtration rate.[1] The reason: no other compound (e.g., mannitol, thiosulfate, urea, creatinine) is completely filtered by the glomeruli without tubular excretion or reabsorption. All you need is a preparation pure enough for intravenous use. *Inulin 10% Warner-Chilcott* is the only commercially available pyrogen-free inulin, controlled to contain less than 0.5% fructose. High molecular weight further increases the accuracy of the test.

the clue: SPC

At New York's Beth Israel Hospital, an old man developed a severe nose bleed while receiving quinidine and bishydroxycoumarin. Either drug might have caused the hemorrhage. Which one did? The clue came from a simplified serum prothrombin consumption (SPC) test, employing *Fibrinogen Warner-Chilcott.* The patient's SPC was 13 seconds (normal: above 30), his plasma prothrombin time (PPT) was 23.7 seconds (normal: 14.2). Since anticoagulants which produce hypoprothrombinemia increase PPT but don't affect SPC, and since the platelet count was also drastically reduced, an abnormality of thromboplastin, rather than prothrombin, was suspected. With cessation of quinidine came rapid clinical and laboratory improvement.[2]

a gentle divorce

"Too bad," our secretary said to us the other day, "seems like the most interesting fellows are always the ones who are married!" Quick as a flash, that reminded us of ketosteroids. Some of them, of course, are free. But the more interesting ones are conjugated; and have to be divorced for assay. The older way of accomplishing this—acid hydrolysis – is rough on some molecules, may change them altogether. On the other hand, glucuronide conjugates can now be digested enzymatically with *Ketodase, Warner-Chilcott's* brand of β-glucuronidase. Ketodase works gently and specifically, leaving the steroid nucleus intact. Ready to use, economical, time-saving, whenever urinary or blood steroids are to be determined.

know how

If you rely on routine hematology to find out whether a patient needs a blood transfusion, your conclusions may be "uniformly, and sometimes wildly wrong."[3] So states a group of Southern physicians, who advocate the *Evans Blue* technic of blood volume determination. They call it an "enormous advance," particularly for the smaller hospital.[3] "This method ... has the advantages of simplicity, availability and accuracy."[4] It is "readily mastered by the average technician."[5] A new, simplified presentation of the *Evans Blue Warner-Chilcott* procedure is now available. It includes precalculated normal values, sample calculations, instructions for common types of photometers. Yours free for the asking.

1. New and Nonofficial Remedies, Philadelphia, J. B. Lippincott Company, 1954, p. 329. 2. Sussman, L. N.; Cohen, I. B., and Gittler, R.: J.A.M.A. *156*:702 (Oct. 16) 1954. 3. Parsons, W. H.; Williams, W. T., and Forsythe, L. J.: Ann. Surg. *135*:791 (June) 1952. 4. Beling, C. A.; Bosch, D. T., and Carter, O. C.: Geriatrics 7:179 (May-June) 1952. 5. Whiting, J. A.; Hotz, R.: Surg., Gynec. & Obst. *97*:709 (Dec.) 1954.

WARNER-CHILCOTT

Laboratory Supply Div., Warner-Chilcott Laboratories
113 West 18th Street, New York 11, N. Y.

Please send me more information on

- ☐ Inulin 10% Warner-Chilcott
- ☐ Ketodase
- ☐ Fibrinogen Warner-Chilcott
- ☐ Evans Blue Warner-Chilcott

NAME_____

ADDRESS_____

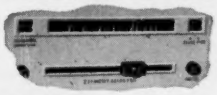
CITY_____ STATE_____

A New Flame Photometer

designed for the people who will use it

1. Thumb-operated wheels give fast, sure control for internal standard measurements; numerical counters permit instant readings.

- **as fast as 50 seconds per analysis**
- **as accurate as ± 1%**
- **as dependable as Perkin-Elmer can make it (leader in the field for 10 years—with 1000 proved instruments)**
- **as simple as dialing a phone**



2. Sliding dial permits quick choice of emission wavelengths.

Perkin-Elmer's new MODEL 146 FLAME PHOTOMETER has been functionally designed to give *performance with convenience*. All controls are grouped at the operator's fingertips. Easy-to-read numerical counters replace vernier dials. Drift-free, vibration-proof electronics give maximum sensitivity and stability. Any gas: natural, city, propane, or acetylene may be used.

A prism monochromator permits (1) analysis of *any* element emitting in the visible, (2) high discrimination, (3) rapid selection.

The MODEL 146 FLAME PHOTOMETER with its attractive, colorful design, its assured performance characteristics—is a valuable addition to any laboratory.

3. Newly designed atomizer-burner system works on any gas, comes apart for easy cleaning.

THE PERKIN-ELMER CORPORATION
NORWALK • CONNECTICUT
Offices in: Chicago, New Orleans, Silver Spring, Md.

LANCASTER PRESS, INC., LANCASTER, PA.